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Advanced studies on phenol removal using *Arthromyces ramosus* peroxidase

by

Daniel Alberto Villalobos Tinoco ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

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Daniel Alberto Villalobos Tinoco
Juan de Oñate # 1136
Col. Las Aguilas, C.P. 78260
San Luis Potosí, S.L.P.
México

Dated August 22, 2000

ABSTRACT

The use of *Arthromyces ramosus* peroxidase (ARP) to remove phenol from aqueous solution in the presence of high molecular weight polyethylene glycol (PEG), a protective additive, was investigated to study the stoichiometry, reactor configuration, and inactivation mechanisms affecting phenol removal.

Attempts to model the stoichiometry based on initial concentration of phenol, hydrogen peroxide, or peroxidase were unsuccessful. However, a tendency towards a 1:1 stoichiometry was observed as the initial ratio (peroxide/phenol) was increased.

Turnovers were independent of the initial enzyme dose, suggesting that step enzyme addition would not increase phenol removal. On the other hand, step addition of hydrogen peroxide over an extended period of time showed an improvement in phenol removal in the reaction mixture.

The results from all of the experiments suggested that the main cause of ARP inactivation in the presence of PEG was produced by the interaction between enzyme and peroxide, creating permanent and non-permanent inactive forms, rather than free-radical or enzyme polymer entrapment inactivation.

If we knew what we were doing,
it wouldn't be called research, would it?

Albert Einstein (1879-1955)

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Advanced studies on phenol removal using *Arthromyces ramosus* peroxidase** by **Daniel Alberto Villalobos Tinoco** in partial fulfillment of the requirements for the degree of **Master of Science** in **Environmental Engineering.**

Ian Buchanan

Dr. I.D. Buchanan, Supervisor

T B Yu

Dr. T. Yu

John Feddes

Dr. J. Feddes

Date **August 21, 2000**

DEDICATION

To my parents, who have always been
by my side, providing me with love,
support, and confidence thru my
education and all the
way in my life.

To my sister, who has been always
there for me, in good and bad moments.

And to those people which, in one way or another,
have influenced in the completion
of these work, either by their
work or support.

Thank you.

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To the other members of the examining committee, Dr. Tong Yu, from the Department of Civil and Environmental Engineering, and Dr. John Feddes, from the Department of Agricultural, Food and Nutricional Sciences for their time and comments to enrich this work.

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1. INTRODUCTION

The removal of phenols and aromatic compounds from contaminated industrial aqueous streams before discharge into any water body is very important since they are toxic, some have been classified as hazardous substances (toxic priority pollutants), and some have been determined to be human carcinogens and mutagens (Klibanov, 1982). These contaminants are present in a variety of wastewater streams generated from industrial applications such as metal casting, wood preservation, coal conversion, pulp and paper mills, petroleum refining, resins, plastics, iron and steel, textile, dyes and organic chemical manufacturing, timber, soaps and detergents, paving and roofing, ore mining and dressing (Klibanov, 1982). The typical concentration of phenol found in industrial wastewater ranges from 1.0 – 10.0 mM (100 to 1000 mg/L) (Al-Kassim *et al.*, 1993; Wu *et al.*, 1993).

Conventional treatment methods such as chemical and physical processes are not suitable for treating moderate to high concentration wastewaters because they are not highly selective, consequently large quantities of oxidant are required to achieve significant removal of these compounds. As a result, the economical feasibility makes these methods more appropriate to treat wastewater with low concentrations or as a final treatment for polishing purposes. Biological processes are not appropriate for treating streams containing high concentrations of phenols because they are inhibitory to mixed cultures (Ibrahim *et al.*, 1997a), and fluctuations in the phenolic load cause operational problems (Allsop, 1993). In addition, industrial effluents can vary greatly in many parameters such as temperature, pH, concentrations of targeted pollutant, levels of other solutes and particulates, even for the same industry, which could limit the selection of a specific treatment method (Nicell *et al.*, 1993a). Thus, to work properly, biological oxidation processes need large capacity ponds for dilution purposes or a long hydraulic retention time for phenol treatment because of the adverse effects on microbes mentioned before (Nakamoto and Machida, 1992). Other existing methods for the removal of

aromatic compounds from wastewater include electrochemical techniques, irradiation, adsorption on activated carbon, extraction, and incineration, but these methods suffer to varying degrees from problems such as high cost, incomplete removal, formation of hazardous byproducts, or low efficiency (Ibrahim *et al.*, 1997b; Klibanov, 1982).

Due to all these complications, a more effective method of aromatic removal was needed, not only because of the disadvantages already mentioned, but also due to the increasing number of new industries, which increases contaminant load to receiving waters. In the 1980s, researchers started to study the ability of peroxidases and other phenol-oxidizing enzymes to remove phenolic contaminants from wastewater (Aitken and Heck, 1998). Peroxidases have the ability to catalyze very effectively the oxidation of complicated organic molecules (George, 1953), such as phenolic compounds. Because of their oxidative properties, peroxidases have many potential uses in the wastewater treatment field, mainly pertaining to industrial wastewater treatment. They can oxidize many colored substances rapidly (Abelskov *et al.*, 1997) and precipitate phenolic compounds, which may be easily removed by sedimentation or filtration (Nicell *et al.*, 1993a; Buchanan and Nicell, 1997; Buchanan *et al.*, 1998).

Peroxidases belong to a specific group of enzymes that contain a metal ion as cofactor, which is a non-protein compound that combines with an inactive protein resulting in a catalytically active enzyme (Bailey and Ollis, 1986). The catalytic ability of the enzymes increases the rate at which a chemical reaction takes place without affecting the reaction equilibrium (Bailey and Ollis, 1986). These catalytic reactions occur in a specific region of the enzyme called *active site*, where the reaction takes place and products are released. Peroxidases are rarely found in the animal kingdom, but are common in plants, involved in the polyaromatic biosynthesis. In the cooxidation of phenols and amines, as peroxide is reduced, phenolic and aromatic amine free radicals are generated (Walsh, 1979). Peroxidase polymerizes phenolic compounds to lignin in plants (Klibanov *et al.*, 1983).

In 1983, Alexander Klibanov and coworkers proposed the use of peroxidase for dephenolization of coal-conversion wastewater, using Horseradish Peroxidase (HRP). Since then many researchers have studied and determined the ability of this peroxidase to treat successfully various industrial wastewaters containing phenols (Klibanov *et al.*, 1983; Klibanov, 1982; Nakamoto and Machida, 1992; Cooper and Nicell, 1996; Buchanan *et al.*, 1997, 1998; Aitken and Heck, 1998; Dec and Bollag, 1994; Maloney, 1984; Al-Kassim *et al.*, 1994). Even tough HRP has been the most studied, peroxidases from *Coprinus cinereus* (CiP), *Coprinus macrorhizus* (CMP), *Arthromyces ramosus* (ARP), and soybean (SBP) have been found to be effective in removing phenol and phenolic compounds from wastewater (Buchanan *et al.*, 1998; Klibanov *et al.*, 1980; Al-Kassim *et al.*, 1993, 1994). All of these peroxidases have shown the ability to have a quick response to shock loadings, an easy on-line addition with a minimal delay compared to biological treatment, since there is not need for biomass growth and acclimation; are effective at low hydraulic retention times and over a broad pH, temperature, and salinity range; operate under less corrosive conditions than chemical oxidation methods; produce less sludge than biological treatment; are effective over a broad range of substrate concentrations; can be applied without dilution; and require very short reaction times (Ibrahim *et al.*, 1997a). Of these peroxidases, that from ARP has attained a lot of attention due to its ease to production, high specific activity, and broad substrate specificity (Abelskov *et al.*, 1997). ARP is isolated from the hypomycete *Arthromyces ramosus* (fungi imperfecti), which produces large amounts of peroxidase extracellularly.

The ability demonstrated by peroxidases to oxidize complicated organic molecules such as phenol or aromatic amines is connected with their ability to form intermediate compounds in the absence of the reducing substrate. Those compounds react rapidly with the reducing agent when it is added. Initially, the native enzyme undergoes a two-electron oxidation by hydrogen peroxide forming an intermediate state called compound I. Then compound I will accept an aromatic compound (phenol) in its active site and will carry out its one electron oxidation, liberating a free radical that is released

back into the solution, and converting compound I to compound II. A second aromatic compound is accepted in the active site of compound II and is oxidized, resulting in the release of a second free radical and the return of the enzyme to its native resting state, completing the enzyme's catalytic cycle (Nicell *et al.*, 1993a; Walsh, 1979, Wu *et al.*, 1999). According to this mechanism, the theoretical stoichiometry between peroxide consumed and aromatic compound oxidized, should reflect a peroxide/phenol ratio of consumption of 1:2. In spite of this, researchers have reported a variety of stoichiometry values for the different peroxidases studied (Buchanan and Han, 2000; Cooper and Nicell, 1996; Nicell, 1994; Nicell *et al.*, 1992, 1994; Al-Kassim *et al.*, 1993, 1994), with a tendency towards unity as a limit, but with no well-established relationship identified.

Disadvantages of enzymatic treatment include the actual elevated cost of producing high purity enzyme isolated from their parent organisms, the disposal of solid products from the reaction, and the possible formation of small amounts of hazardous soluble byproducts (Nicell *et al.*, 1993a). The use of high purity enzymes is preferred over the intact organisms because they are highly specific for their substrates; they are easier to store and handle; concentration is easier to control; their potency can be better standardized; and enzyme concentration is not dependent on bacterial growth rates (Ibrahim *et al.*, 1997a; Nicell *et al.*, 1993b). The possible by-products formed during polymerization depend on the parent phenol, the source of enzyme, and the reaction pH (Aitken *et al.*, 1994). However, Aitken and coworkers (1994) studied and analyzed the byproducts formed during enzyme-catalyzed reaction of phenol, determining that their toxicity may be minimized by controlling the reaction conditions.

In addition, various side reactions take place, some of which are responsible for enzyme inactivation or inhibition, causing a limited catalytic lifetime (Ibrahim *et al.*, 1997a; Nicell *et al.*, 1993b). A number of theories have been proposed related to enzyme inactivation. Enzyme inactivation or inhibition by hydrogen peroxide (with limited or no aromatic substrates present) results in the formation of compound III, which is a catalytically inactive form of the enzyme (Buchanan and Han, 2000; Ibrahim *et al.*,

1997b), but does not represent a terminal inactivation, since compound III decomposes back to peroxidase (Arnao *et al.*, 1990a). The peroxidases HRP, ARP, CiP, CMP, and SBP have shown similar conversion to compound III in a time and H_2O_2 dependent manner. Moreover, a permanent inactive form of HRP designated P-670 because of its characteristic spectral peak absorbance at a wavelength of 670 nm, has been observed with HRP. In addition to these side reactions, other enzyme inactivation mechanisms have been proposed. Several studies have indicated that the apparent inactivation of HRP occurs during phenol polymerization, where the end-product polymer adheres to peroxidase molecules enclosing them, thus hindering the access of substrate to the enzyme's active site (Klibanov *et* al., 1983; Nicell *et al.*, 1993b; Nakamoto and Machida, 1992; Tatsumi *et al.*, 1994). This was inferred by Nakamoto and Machida (1992) since traces of peroxidase (HRP) initially dissolved in the reaction mixture were removed along with the polymerized phenol by centrifugation. Klibanov *et al.* (1983) speculated that a free radical may return to the active site of the enzyme and bond to the heme edge causing permanent enzyme inactivation (Ibrahim *et al.*, 1997b; Nicell *et al.*, 1993a), by blocking the access to the active site or upsetting the geometric configuration of the enzyme, eliminating its catalytic ability (Nicell *et al.*, 1992, 1993a; Bailey and Ollis, 1986; Wright, 1995; Buchanan and Nicell, 1997). It is likely that both of these mechanisms contribute to permanent enzyme inactivation.

In order to slow enzyme inactivation, different chemical additives such as gelatin, high molecular weight polyethylene glycol (PEG), talc, proteins, hydrophilic polymers such as milk casein, bovine serum albumin, and polyvinyl alcohol have been studied. Of these additives, PEG has been found to be the most viable (and the most studied) since it is not toxic to humans, has the less negative effects on the effluent and no centrifugation is required to remove the products formed. Although some improvements have been achieved by using additives in the reaction mixture, inactivation still occurs, hampering aromatic removal. The main reason that inactivation cannot be completely avoided is due to the incomplete understanding of the inactivation mechanisms that happen during the enzymatic reaction.

1.1. Objective

The objective of this research is to improve the understanding of the stoichiometric relationship between phenol removal and peroxide consumption occurring during the reaction, and to study the inactivation mechanisms of *Arthromyces ramosus* peroxidase in the presence of PEG.

1.2. Scope

The scope of this research comprises:

- Study and modeling attempt of stoichiometry.
- Characterization of *Arthromyces ramosus* peroxidase inactivation.
- Investigation of reactor operation to reduce inactivation.

2. LITERATURE REVIEW

2.1. Enzymes

Arthromyces ramosus peroxidase (ARP), as other enzymes, is a protein catalyst with a molecular mass between 38,000 and 41,000 Daltons (Farhangrazi *et al.*, 1994; Kjalke *et al.*, 1992), belonging to the Class II heme-containing peroxidases (secretory fungal peroxidases) (Smulevich, 1998).

Enzymes act as catalysts. A catalyst increases the rate at which a chemical reaction takes place, but without affecting the reaction equilibrium. The enzyme does not undergo a permanent chemical change (Bailey and Ollis, 1986).

In an uncatalyzed reaction, the activation energy required to react is high, whereas a catalyst lowers the activation energy required, permitting molecules with smaller internal energies to react. Notice that the standard free energy remains constant either with the use of a catalyst or not (refer to Figure 2-1).

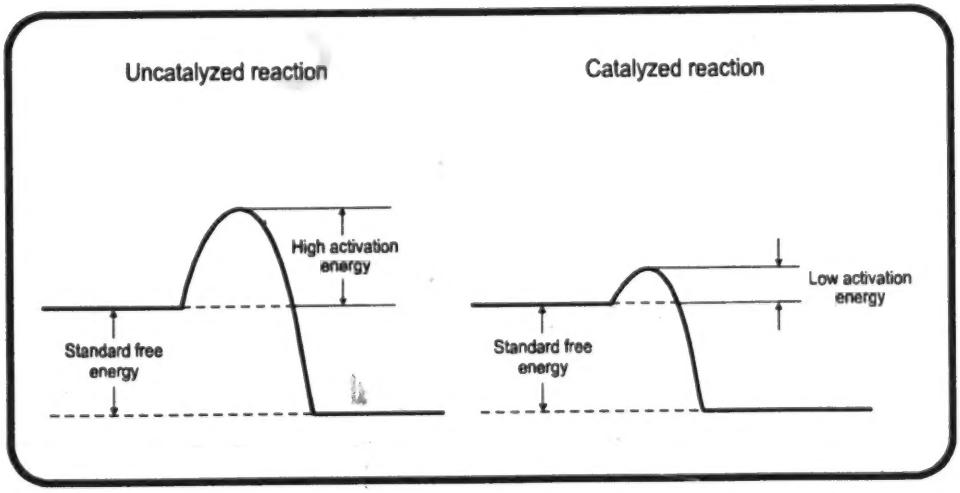

Figure 2-1. Activation energy in an uncatalyzed and catalyzed reaction.

The catalytic reactions occur in a specific region of the enzyme called *active site*, where the reaction takes place and products are released. In this site, the protein molecule is folded in a particular way where a certain group of reactive amino acid side chains present a very specific site to the substrate. Because the number of reactive groups near the substrate is typically 20, which is far less than the total number of groups present in the enzyme, it is believed that only a small part of the enzyme acts as an active site. The remaining amino acids determine the folding of another chain of amino acids, that is called the secondary structure, and the disposition of one part of a folded chain next to another, a tertiary structure, which helps to create the active site (Bailey and Ollis, 1986).

It is important to note the difference between an enzyme and a synthetic catalyst. Most synthetic catalysts will catalyze a variety of reactions involving many different kinds of reactants (or substrates); this means that they are not specific. On the other hand, while enzymes are not very specific, many enzymes will catalyze only one reaction involving only specific substrates. This specificity depends upon the enzyme's biological role (Bailey and Ollis, 1986).

Another difference between enzymes and synthetic catalysts is that enzymes frequently need a *cofactor*. A cofactor is a non-protein compound (called *apoenzyme* by biochemists), which combines with an inactive protein resulting in a catalytically active enzyme (Bailey and Ollis, 1986).

Two types of cofactors exist: (1) a metal ion, which is the simplest; and (2) a *coenzyme* that is a complex organic molecule (Bailey and Ollis, 1986).

There is a particular group of enzymes containing a metal ion as cofactor, called ferri-(Fe^{III})-hemoprotein that have been termed *hydroperoxidases*. Two groups of hydroperoxidases exist: *catalases* preferring H_2O_2 (hydrogen peroxide) as substrate, and *peroxidases* preferring alkyl peroxides, albeit each type can use both as substrate (Walsh, 1979). The latter enzyme catalyzes very effectively the oxidation of complicated organic

molecules (George, 1953). Many researchers have studied and determined the ability of this enzyme to treat successfully various industrial wastewaters containing phenols (Klibanov *et al.*, 1983; Klibanov, 1982; Nakamoto and Machida, 1992; Cooper and Nicell, 1996; Buchanan *et al.*, 1997, 1998; Aitken and Heck, 1998; Dec and Bollag, 1994; Maloney, 1984; Al-Kassim *et al.*, 1994).

Among the peroxidase enzymes, those from horseradish (HRP), *Coprinus cinereus* (CiP), *Coprinus macrorhizus* (CMP), *Arthromyces ramosus* (ARP), and soybean are effective in removing phenol and phenolic compounds from wastewater (Buchanan *et al.*, 1998; Klibanov *et al.*, 1980; Al-Kassim *et al.*, 1993, 1994).

Peroxidases are rarely found in the animal world, but are common in plants, involved in the polyaromatic biosynthesis. In the cooxidation of phenols and amines, as peroxide is reduced, phenolic and aromatic amine free radicals are generated (Walsh, 1979). This enzyme (peroxidase) polymerizes phenolic compounds to lignin in plants (Klibanov *et al.*, 1983).

Peroxidases are Fe^{III} hemoproteins in the resting state. They are brown in color and show characteristic optical spectra with α, β and Soret bands around 600, 500, and 400 nm, respectively (Walsh, 1979).

Factors affecting or influencing an enzyme's catalytic activity, presumably by affecting the enzyme's shape or ionization state are (Bailey and Ollis, 1986):

1. pH
2. Temperature
3. Fluid Forces (shear stress and hydrostatic pressure)
4. Chemical agents (such as alcohol and urea)
5. Irradiation (light, sound, ionizing radiation)

2.2. Use of Peroxidase as an Alternative Method for Treating Phenolic Wastewaters

Aromatic compounds, mainly phenols and aromatic amines, are present in wastewater from industrial applications such as metal casting, wood preservation, coal conversion, pulp and paper mills, petroleum refining, resins, plastics, iron and steel, textile, dyes and organic chemical manufacturing, timber, soaps and detergents, paving and roofing, ore mining and dressing (Klibanov, 1982). The typical concentration of phenol found in industrial wastewater ranges from 1.0 – 10.0 mM (100 to 1000 mg/L) (Al-Kassim *et al.*, 1993; Wu *et al.*, 1993).

The importance of removing this pollutant from aqueous streams before discharge into any water body, is because (1) they are toxic; (2) some have been classified as hazardous substances (toxic priority pollutants); and (3) and some have been determined to be human carcinogens and mutagens (Klibanov, 1982).

In the 1980s, researchers started to study the ability of using peroxidases and other phenol-oxidizing enzymes to remove phenolic contaminants from wastewater (Aitken and Heck, 1998). In 1983, Alexander Klibanov and coworkers proposed the use of peroxidase for dephenolization of coal-conversion wastewater, using Horseradish peroxidase (HRP).

Since then, many other researchers have studied HRP and other peroxidases for the removal of various pollutants. These studies have included investigation of the use of various reactor configurations, the mechanism of removal, enzyme inhibition and inactivation, among other aspects of the removal process.

Because of its oxidative properties, peroxidase has many potential uses in the wastewater treatment field, mainly pertaining to industrial wastewater treatment. It can

oxidize many colored substances rapidly, therefore having a great potential in the textile, paper and pulp bleaching industries (Abelskov *et al.*, 1997).

In cases where only a specific class of contaminant is to be removed, conventional treatment methods such as chemical and physical processes are not suitable for treating moderate to high concentration wastewaters because they are not highly selective. Thus, large quantities of oxidant (or adsorbent) are required. These methods are more appropriate to treat wastewater with low concentrations or as a final treatment for polishing purposes. Biological processes are not appropriate for treating streams containing high concentrations of phenols because they are inhibitory to mixed cultures (Ibrahim *et al.*, 1997a), and fluctuations in the phenolic load cause operational problems (Allsop, 1993). To work properly, biological oxidation needs either large capacity ponds for dilution purposes or a long hydraulic retention time for phenol treatment due to the adverse effects on microbes mentioned above (Nakamoto and Machida, 1992).

The enzymatic removal of phenols from aqueous streams starts when enzyme and hydrogen peroxide are added to the polluted water. Immediately, the enzyme is oxidized (or activated) by hydrogen peroxide. Once activated, the enzyme passes through two catalytically active forms called compound I and compound II before returning again to its native form. Each one of these intermediate states oxidizes one molecule of phenolic substrate (AH_2) in the active site producing a phenoxy radical (also known as free radical - $AH^{\bullet}$). Then, the free radicals are released back into the solution where they couple forming insoluble polymer products, which eventually may be easily removed from the solution by sedimentation or filtration.

Industrial effluents can vary greatly in many parameters such as temperature, pH, concentrations of targeted pollutant, levels of other solutes and particulates, even for the same industry, which could limit the selection of a specific treatment method (Nicell *et al.*, 1993a). Conversely, enzymes (as it will be mentioned later in more detail) achieve satisfactory phenol removal over a broad pH and temperature ranges. In addition,

industrial wastewater may contain a mixture of pollutants that have to be removed. If a few of these pollutants can be easily precipitated by the use of peroxidase, those few will facilitate the removal of others by a coprecipitation effect (Klibanov, 1982). Furthermore, Klibanov (1983) observed that easy to remove phenols helped in the enzymatic precipitation of hard to remove phenols and amines, apparently because the phenoxy radicals formed from the easily removed phenols can react with, and coprecipitate the hard to remove ones, or the harder to remove contaminant adsorbs on the precipitate formed by the easier to remove contaminant.

Contrasting all the advantages mentioned above, some disadvantages to the process of enzyme catalyzed polymerization include high catalyst cost, disposal of solid products from the reaction, and the possible formation of small amounts of hazardous soluble byproducts (Nicell *et al.*, 1993a). Another important factor to consider before applying this methodology in full-scale systems is the formation of soluble and insoluble products during polymerization. These products vary in molecular mass and composition depending on the parent phenol, the source of enzyme, and the reaction pH (Aitken *et al.*, 1994). In consequence, the nature of the products in treating a specific wastewater cannot be predicted. However, Aitken and coworkers (1994) studied and analyzed the byproducts formed during enzyme-catalyzed reaction of phenol, determining that their toxicity may be minimized controlling the reaction conditions.

Studies performed by Alberti and Klibanov (1982) and by Cooper *et al.* (1996) indicate that the use of crude or high purity enzyme have the same treatment efficiency. However, isolated enzymes from their parent organisms are preferred over the intact organism because they are highly specific for their substrates, they are easier to store and handle, concentration is easier to control, their potency can be better standardized, and enzyme concentration is not dependent on bacterial growth rates (Ibrahim *et al.*, 1997a; Nicell *et al.*, 1993b). The only disadvantage in using pure enzymes is the high cost of them, because of the sophisticated methods of purification and the currently limited demand (Cooper and Nicell, 1996). Despite this high cost of enzyme, according to

Klibanov and Morris (1981), the use of purified enzyme is comparable in price to the peroxide treatment of industrial wastewaters in the presence of an iron catalyst (Nicell *et al.*, 1993a).

Electrochemical techniques, irradiation, adsorption on activated carbon, extraction, and incineration are other existing methods for the removal of aromatic compounds from the wastewater, but these methods suffer to varying degrees from problems such as high cost, incomplete removal, formation of hazardous byproducts, or low efficiency (Ibrahim *et al.*, 1997b; Klibanov, 1982).

Enzymatic treatment methods are not restricted to wastewater treatment. They can also be used for drinking water treatment, since some phenolic compounds arise from natural and domestic sources, and some phenolic compounds present taste and odor problems, especially in the presence of chlorine, where mono and dichlorophenol may be formed (Maloney, 1984).

2.3. Peroxidase Cycle

George (1953) and Chance (1952) first described the peroxidase cycle. During their research, some other mechanisms were thought to be acting on the general oxidation-reduction reactions involved. George stated that the ability demonstrated by peroxidases in oxidizing complicated organic molecules such as phenol or aromatic amines is connected with their ability to form intermediate compounds in the absence of the reducing substrate. Those compounds react rapidly with the reducing agent when it is added. Three compounds are identifiable based on their particular color and absorption spectrum: these are the resting state of the enzyme, as well as compounds I, and II as shown in the following reactions.

$$E + H_2O_2 \xrightarrow{k_1} E_i + H_2O \qquad (2.1)$$

$$E_i + AH_2 \xrightarrow{k_2} E_{ii} + AH^{\bullet} \qquad (2.2)$$

$$E_{ii} + AH_2 \xrightarrow{k_3} E + AH^{\bullet} + H_2O \qquad (2.3)$$

where, E, E_i, and E_{ii} represent the enzyme's ground state, compound I and compound II forms, respectively.

With the overall reaction being as follows:

$$H_2O_2 + 2AH_2 \xrightarrow{enzyme} 2AH^{\bullet} + 2H_2O \qquad (2.4)$$

In equation (2.1) the native enzyme (ferric, Fe^{III}) undergoes a two-electron oxidation by hydrogen peroxide from the resting state to an intermediate state called compound I (ferryl, Fe^{V}), with the release of one molecule of water. Then compound I will accept an aromatic compound (phenol) in its active site and will carry out its oxidation (accepting an electron), resulting in a free radical that is released back into the solution. At the same time, compound I is reduced by one electron to compound II, having a formal oxidation state Fe^{IV} (ferrous) (equation 2.2). A second aromatic compound is accepted in the active site and is oxidized now by compound II, and a second free radical is released. A molecule of water is also released and compound II is reduced back to the enzyme's native resting state (equation 2.3) completing the enzyme's catalytic cycle (Nicell *et al.*, 1993a; Walsh, 1979, Wu *et al.*, 1999).

In the overall equation (2.4) it can be appreciated that for each mole of hydrogen peroxide consumed, two aromatic substrate molecules are oxidized to free radicals and two molecules of water are released.

Substrates known to reduce compound I and compound II include various phenols, aromatic amines, and polyphenols (Wright, 1995).

As mentioned before, two donor molecules (AH_2) from the reducing substrate are released as free radicals (AH· - phenoxy radicals) from the active site of the enzyme into the solution, spontaneously reacting non-enzymatically forming dimers, which are less soluble than their monomer precursors. If these dimers formed after the reaction fail to precipitate from the solution, they still have the potential to undergo further oxidation by being accepted again in the active site of the enzyme, resulting in the formation of trimers, tetramers, or higher molecular mass polymers that are even less soluble. These high mass polymers eventually precipitate and can be more easily removed by filtration or sedimentation (Nicell *et al.*, 1993b, 1994, 1996; Ibrahim *et al.*, 1997a; Wu *et al.*, 1993).

The polyaromatic products form according to:

$$2\,AH^{\bullet} \rightarrow A_2H_2 \qquad (2.5)$$

Equations (2.1), (2.2), and (2.3) depict the catalytic cycle of peroxidase. Apart from these, side reactions also take place, some of which are responsible for enzyme inactivation or inhibition, causing a limited catalytic lifetime (Ibrahim *et al.*, 1997a; Nicell *et al.*, 1993b).

This catalytic lifetime is also termed "turnovers" that is defined as the number of aromatic molecules precipitated per molecule of enzyme consumed, representing the number of times that the enzyme carries out its catalytic cycle before being inactivated (Ibrahim *et al.*, 1997a; Nicell *et al.*, 1993b). It is important to remark that the term "turnovers" is not the same as the term used in enzymology, "the turnover number", which is the number of moles of substrate converted per minute per mole of enzyme

under saturating conditions (Wu *et al.*, 1993). Turnovers, according to Nicell (1994), are calculated as:

$$Turnovers = \frac{[AH_2]_0 - [AH_2]_f}{E_0} \qquad (2.6)$$

where $[AH_2]_0$ is the initial aromatic substrate in the reactor; $[AH_2]_f$ is the aromatic substrate concentration after complete reaction; and E_0 is the initial native enzyme concentration (all expressed in moles, M), which is calculated according to:

$$E_0 = C_a \times Activity\ (U/mL) \qquad (2.7)$$

where C_a [$M \cdot mL \cdot U^{-1}$] its a constant of proportionality between the molar concentration of active enzyme and the enzyme activity, which are directly proportional.

This catalytic lifetime depends upon the combination of mechanical, chemical, and thermal processes and their interaction (Ibrahim *et al.*, 1997a). At elevated temperatures and extreme pH, the enzyme is susceptible to perturbations in geometry, chemical structure, or both, diminishing its catalytic ability.

An enzyme-catalyzed reaction where a transfer of a group from a donor to the enzyme followed by a second transfer from enzyme to acceptor is known as substitued-enzyme or ping-pong mechanism. This mechanism involves a two-substrate two-product scheme, where the enzyme passes through a single intermediate form before returning to its native state. In the peroxidase case, the enzyme cycle is defined as a modified ping-pong mechanism, since the cycle involves the production of three products (water and two free radicals, H_2O and 2 AH·) and the enzyme passes through two intermediates, compound I and compound II (Nicell, 1994).

2.4. Side Reactions

As mentioned before, a number of side reactions have been found to limit the enzyme's catalytic life. Figure 2-2 represents the enzyme's cycle, including some known side reactions.

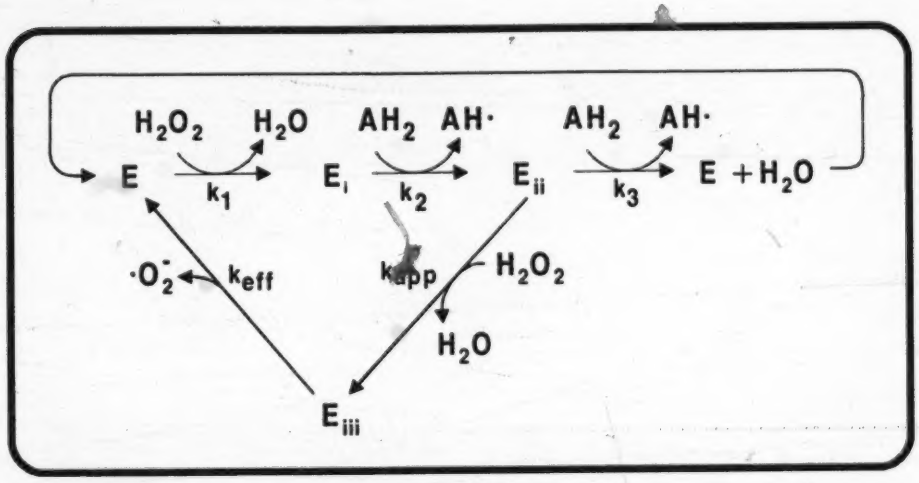

Figure 2-2. Main reactions of peroxidase cycle in the presence of aromatic substrate.

One of these side reactions is the oxidation of compound II by excess hydrogen peroxide into a third intermediate, named compound III (E_{iii}). This compound is also called oxyperoxidase because it is formed directly from a reaction of the ferrous enzyme with oxygen. Compound III is a catalytically inactive form of the enzyme (Buchanan and Han, 2000; Ibrahim *et al.*, 1997b) and it is described by the following mechanism:

$$E_{ii} + H_2O_2 \xrightarrow{k_{app}} E_{iii} + H_2O \qquad (2.8)$$

$$E_{ii} + H_2O_2 \rightarrow (E_{ii} \cdot H_2O_2) \rightarrow E_{iii} + H_2O \qquad (2.9)$$

where E_{iii} represents the compound III form of the enzyme.

Equation (2.9) represents the theoretical conversion of compound II to compound III, but the existence of the middle form $(E_{ii} \cdot H_2O_2)$ has not yet been confirmed (Nicell, 1994). Consequently equation (2.8) has been accepted as a valid mechanism. Nakajima and Yamazaki (1987), and Adediran and Lambeir (1989), have reported apparent rate constants (k_{app}) for the formation of compound III from compound II at neutral pH and various temperatures.

Compound III has a structure resonating between Fe(II)-O_2^- and Fe(III)-O_2^- (Wright, 1995). An oxidation state of VI has been assigned to compound III (Wright, 1995, Adediran and Lambeir, 1989).

Compound III of ARP seems to have a characteristic spectra, showing three peaks at 418, 549, and 582 nm (Buchanan and Han, 2000), similar to that found by Andersen *et al.* (1991) for *Coprinus cinereus* peroxidase (CiP), which is deemed to be virtually identical to ARP. These researchers (Andersen *et al.*, 1997; Kjalke *et al.*, 1992) reported CiP to absorb most strongly at 417, 547, 582, and 649 nm. No peak is observed at 649 nm in the case of ARP.

Formation of compound III does not represent a terminal inactivation, since compound III decomposes back to native peroxidase according to (Arnao *et al.*, 1990b):

$$E_{iii} \rightarrow E + O_2^- \qquad (2.10)$$

Although the formation of this inactive enzyme intermediate is not permanent, the return to the native enzyme state is sufficiently slow to severely inhibit the catalytic ability of the enzyme to oxidize aromatic substrates. Thus, any accumulation of compound III represents a loss in catalytic ability (Ibrahim *et al.*, 1997a; Nicell *et al.*, 1993a). Buchanan and Han (2000) observed that the representative peaks shown by compound III disappeared within 20 minutes, and the peak wavelength reverted to 407 nm, indicating the conversion of compound III back to native enzyme. However, the

spectral scan indicated that compound III was not completely converted to the resting state, but seemingly to an inactive form which absorbs light most strongly outside of the limits of the scan used during their study.

Other peroxidases besides HRP, such as ARP, CiP, and CMP, have shown similar conversion to compound III in a time and H_2O_2 dependent manner, and in the absence of donor substrate (Al-Kassim *et al.*, 1993). Nevertheless, the formation of compound III is different between peroxidases.

Other side reactions observed for HRP include those where in the absence of a reducing substrate and in the presence of excess of H_2O_2, compound I can react with hydrogen peroxide to form an intermediate complex $(E_i \cdot H_2O_2)$, which can react in one of the following routes (refer to Figure 2-3) (Arnao *et al.*, 1990b):

$$E_i + H_2O_2 \underset{k_{-2}}{\overset{k_2}{\leftrightarrow}} (E_i \cdot H_2O_2) \qquad (2.11)$$

$$(E_i \cdot H_2O_2) \overset{k_3}{\rightarrow} E + O_2 + H_2O \qquad (2.12)$$

$$(E_i \cdot H_2O_2) \overset{i}{\rightarrow} E_{670} \qquad (2.13)$$

$$(E_i \cdot H_2O_2) \overset{k_4}{\rightarrow} E_{ii} + O_2^- \qquad (2.14)$$

Reactions (2.11) and (2.12) are weak catalase reactions where the peroxide acts as a reducing agent, converting peroxide into oxygen and water. Reaction (2.11) will be minimal if a stronger reducing agent is present (i.e. phenol).

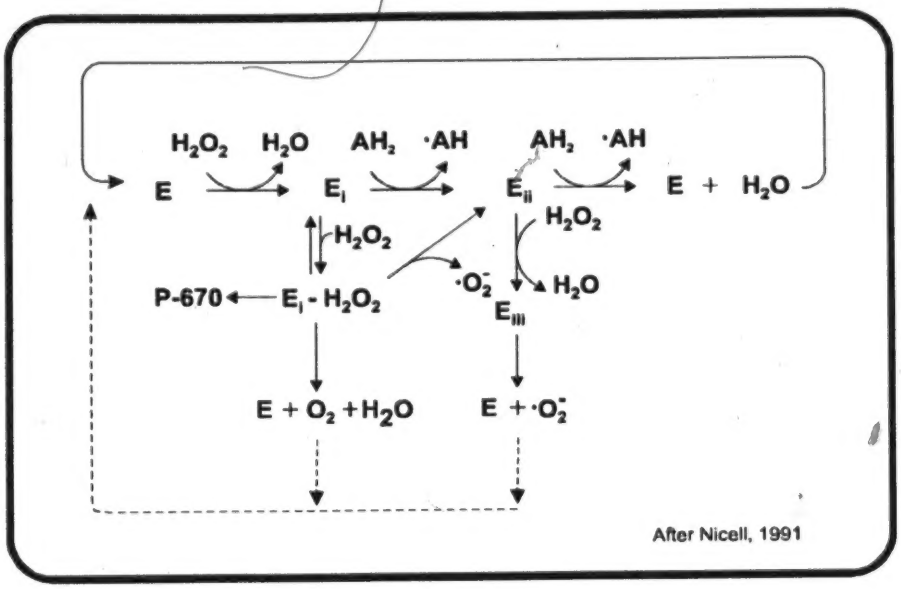

Figure 2-3. Complete peroxidase cycle with suspected side reactions.

The values for rate constants k_2, k_{-2}, and k_3 are not directly known, but the observed rate constant k_c for the reaction (Nicell, 1994):

$$E_I + H_2O_2 \xrightarrow{k_c} E + O_2 + H_2O \qquad (2.15)$$

was measured by Nakajima and Yamazaki (1987) over a range of temperatures. Wright (1995) reported a second order rate constant of 500 $M^{-1}s^{-1}$ at 5°C and an activation energy of 8.0 kcal/mol for HRP in 50 mM pH 7.0 potassium phosphate buffer.

Reactions (2.11) and (2.13) depict the conversion of peroxidase to a permanent inactive enzyme state called verdohaemoprotein, also known as P-670 or P_{670} (Nicell, 1994) because of its characteristic spectral peak at a wavelength of 670 nm.

2.5. Enzyme Inactivation

Enzyme inactivation is a very important factor when evaluating the feasibility of using this treatment in full-scale. The actual high cost of production and purification of the enzyme, jointly with its tendency to inactivate, reduce the cost-effectiveness of enzymatic treatment at present, but studies are underway to understand the various inactivation mechanisms involved and the way to reduce or eliminate them.

Enzyme inactivation can occur from different sources: chemical species can combine with the enzyme altering, inhibiting or destroying its catalytic ability (chemical denaturation); the temperature and pH of the reaction environment can either decrease the probability of certain types of interactions or alter the protein structure of the enzyme inducing inactivation (Nicell *et al.*, 1993a).

There are a number of theories relating to enzyme inactivation. Enzyme inactivation or inhibition based on hydrogen peroxide (with limited or no aromatic substrates) was described in the previous section, while this section is focused on possible permanent inactivation of the enzyme by reaction products.

Klibanov *et al.* (1983) reported that inactivation of HRP takes place during the enzymatic reaction, since no inactivation was observed by phenol nor PEG alone. Apparent enzyme inactivation during phenol polymerization occurs mainly when the end-product polymer adheres to peroxidase molecules (while they are still active) and then they are apparently inactivated. The probable reason is that the polymerized phenol adsorbed the enzyme molecules enclosing them, thus hindering the access of substrate to the enzyme's active site (Nicell *et al.*, 1993b; Nakamoto and Machida, 1992; Tatsumi *et al.*, 1994). This was inferred by Nakamoto and Machida (1992) since traces of peroxidase (HRP) initially dissolved in the reaction mixture were removed along with the polymerized phenol by centrifugation.

Another theory is that a free radical returns to the active site of the enzyme and bonds to the heme edge causing permanent enzyme inactivation (Ibrahim *et al.*, 1997b; Nicell *et al.*, 1993a). Such bond can block the access to the active site or upset the geometric configuration of the enzyme, eliminating its catalytic ability (Nicell *et al.*, 1992, 1993a; Bailey and Ollis, 1986; Wright, 1995; Buchanan and Nicell, 1997). No data are available to describe the mechanisms and kinetics of this kind of inactivation (Nicell, 1994). This mechanism was depicted by Buchanan and Nicell (1997) as follows:

$$Enzyme_{active} + \cdot AH \xrightarrow{k_i} Enzyme_{inactive} \qquad (2.16)$$

where $Enzyme_{active}$ represents all forms of the enzyme that have not been permanently inactivated (this includes compound III) whereas $Enzyme_{inactivated}$ represents permanently inactivated enzyme.

Nicell and coworkers (1992, 1993b) found that reducing the probability of a free radical finding and reentering the active site of an enzyme was possible by reducing the concentration of free radicals in the solution, and the available amount of enzyme for inactivation. This can be achieved by the continuous addition of HRP in small quantities over an extended period, thus reducing the instantaneous concentration of free radicals and enzyme in the reaction mixture. Additionally, the probability that an aromatic molecule had already occupied the active site would increase since the aromatic substrate would be present in relatively higher concentration with respect to the free radicals.

Al-Kassim *et al.*, (1994) confirmed this decrease in enzyme inactivation during their research dealing with CMP, where the results suggested that at high enzyme concentrations, the phenoxy radicals may find an enzyme more readily. Also they mentioned that as the reaction progresses, the concentration of phenol, and hence free radicals, decreases and it is more difficult for the free radicals to find each other, thus increasing the probability of a free radical reentering to an active site. Klibanov *et al.* (1983) also reported similar results concerning removal efficiency, which was higher

when the enzyme was added incrementally rather than all at once, independent of whether the mixture was shaken, stirred or left undisturbed.

Another approach to this issue has been addressed by Aitken and Heck (1998), and Nakamoto and Machida (1992). They proposed a controlled addition of peroxide to the reactor, which not only would decrease the concentration of free radicals in the solution diminishing the possibility of a free radical blocking the active site, but would also diminish any peroxide-dependent mechanism of inactivation. However, Al-Kassim and coworkers (1993) after testing different reactor configurations (e.g. discontinuous addition of enzyme and H_2O_2 in different approaches) reported that only a slight improvement in phenol removal was achieved when both peroxide and ARP were added discontinuously at three different doses over a period of 30 minutes.

A third way of losing enzyme activity is by the reduction of the enzyme quantity present in the reaction. Adsorption of the enzyme onto polymers or entrapment of the enzyme within flocs of solids particles when the polymers formed begin to precipitate promote co-precipitation of the enzyme with them (Nicell *et al.*, 1993b; Buchanan and Nicell, 1997; Buchanan *et al.*, 1998). Wu *et al.* (1999), after making total organic carbon measurements, found that HRP inactivated by the polymer was connected chemically or physically with the polymer. HRP inactivated in the form of verdohaemoprotein (P-670) presumably remains in the solution (Wu *et al.*, 1999).

2.6. Chemical Additives

Chemical additives such as gelatin, high molecular weight polyethylene glycol (PEG), talc, proteins, hydrophilic polymers such as milk casein, bovine serum albumin, and polyvinyl alcohol have been used to reduce the enzyme inactivation caused by the different mechanisms mentioned in the two previous sections. In theory, based on the entrapment of the enzyme into the polymers formed, these additives may have greater

affinity than the enzyme for the hydroxyl groups, and preferably attach to the polymers allowing the enzyme to remain in the solution avoiding its consequential inactivation (Cooper and Nicell, 1996).

Of all these different additives, PEG has been found to be the most viable (and the most studied) since it is not toxic to humans, has the less negative effects on the effluent, and no centrifugation is required to remove the products formed, as with gelatin. Gelatin also contains a high nitrogen content (18% by weight), which may adversely increase the nitrogen levels in treated water. PEG is relatively inexpensive and has a major impact on treatment cost, reducing the enzyme requirements (Cooper and Nicell, 1996). A disadvantage of the PEG is that overdosing can increase the organic loading in the effluent, so its concentration should be minimized (Buchanan and Han, 2000).

Nevertheless, the higher the molecular weight of the PEG added the higher was the degree of protection against inactivation provided. These results came from experimentation with different molecular weight PEG obtained by Nakamoto and Machida (1992), who reported that PEG with average molecular weight under 400 was ineffective in suppressing peroxide activity.

Nakamoto and Machida (1992) observed that when PEG-1000 (molecular weight 1000) is added to the reaction mixture, it is adhered to the polymerized phenol, since only 30% of the initial amount added remained in the supernatant at the end of the reaction. They concluded that PEG adheres to the polymerized phenol instead of the enzyme, which remains in the solution reducing the amount needed for phenol removal. Based on these results they noted that if the enzyme inactivation mechanism were solely due to the free radicals blocking the entrance of substrate to the active site, PEG would be able to provide a minimal or no protective effect over the enzyme. So they suggest that the main mechanism of inactivation is the entrapment or adsorption of peroxidase molecules by polymerized phenol, as previously discussed.

The application of PEG to the reaction mixture enhances the efficiency of the catalytic reaction, having a significant protective effect on the enzyme. Ibrahim *et al.* (1997a, 1997b) observed an improvement of 28 and 70% phenol removal using optimal PEG concentration for 1 and 10 mM, respectively.

The effect of this additive is purely a protective action over the enzyme, and does not directly remove phenol, as shown by Ibrahim and coworkers (1997) in their research, where PEG alone or PEG and H_2O_2 did not remove any phenol from the solution (Ibrahim *et al.*, 1997a). Also Buchanan and Han (2000) and Buchanan and Nicell (1998) showed that PEG had no effect on ARP or HRP activity. The additive is believed to suppress only the rate of permanent inactivation.

Ibrahim *et al.* (1997b) studied the relationship between ARP concentration and the optimal quantity of PEG required to achieve at least 75% phenol removal, concluding that there is a linear relationship between these two parameters. The equation fitted to the data is (with standard deviation of 0, and $r^2 = 1.0$):

$$PEG_{min} = 20 + 10C_{ph} \qquad (2.17)$$

where C_{ph} (mM) stands for phenol concentration in the solution, and PEG_{min} (mg/L) is the optimal PEG dose required.

Wu *et al.* (1993) also measured the improvements in phenol removal for two different concentrations, with and without PEG, using HRP. Their results were confirmed by Tatsumi *et al.* (1994) who demonstrated that the addition of PEG greatly increased the turnovers.

From these results Wu *et al. (1993)* concluded that (1) HRP was effectively protected by the addition of PEG, and (2) the higher the concentration of phenol, the more effective was the addition of PEG. They also found that if the minimal dose of PEG

is not supplied, even an increase in enzyme would not improve the removal efficiency noticeably.

Wu *et al.* (1993) developed an equation similar to (2.17) for the minimal dose of PEG required, but in this case for HRP and phenol concentrations of 1, 3, 5, 7 and 10 mM (for at least 95% removal), with a molar ratio of H_2O_2 to phenol equal to one, using the least-square method, resulting in the following expression (with standard deviation of 3.8 mg/L and $r^2 = 0.9985$):

$$^{1}\, PEG_{min} = 6.5 + 24.1\, C_{ph} \qquad (2.18)$$

Based on equation (2.17) and (2.18) it can be regarded that the minimal dose of PEG is higher for HRP than for ARP, even though the removal efficiencies were set differently.

These empirical models are of great importance from the economical point of view, since any addition above the optimal PEG dose will not be reflected in any further improvement on the removal efficiency (Ibrahim *et al.*, 1997b). This means that if a PEG dose less than the optimal is applied into the reaction mixture, a larger quantity of enzyme will be inactivated. An attempt to increase the removal by increasing the enzyme would be fruitless since the use of more enzyme with a low PEG concentration does not markedly improve the removal efficiency, as observed by Wu *et al.* (1993), where in the presence of less than optimal PEG dose, the increase on enzyme concentration (from 0.05 to 0.2 U/mL) could not achieve 95% removal.

Wu *et al.* (1999) observed that in the absence of PEG, enzyme inactivation rates behave identically to those when PEG had been provided, but consumed as the reaction continued. When the PEG was fully consumed form the reaction mixture the rate of

[1] Equation (2.18) has been modified from its original units (g/L) to (mg/L) in order to visualize easily the difference between equations (2.17) and (2.18).

enzyme inactivation increased dramatically, and the phenol conversion rates rapidly approached zero.

Although enzyme-catalyzed treatment still entails high cost, the use of chemical additives, such as PEG, makes it economically competitive with other processes (Wu *et al.*, 1993).

Buchanan and Han (2000) observed that PEG improves ARP efficiency, but to a lower extent than that for HRP, since rapid inactivation, even with excess of PEG, was observed, contrarily to the improved protective action for HRP. This suggests that the inactivation mechanisms involved are different for these two enzymes.

The use of coagulants to improve phenol removal has also been investigated. Tatsumi *et al.* (1994) proposed the use of a coagulant for aiding the precipitation of the polymerized phenol produced during the enzyme-catalyzed reaction. They experimented with two types of coagulant: (1) cationic polymer coagulants containing amino groups such as hexamethylenediamine epichlorohydrin polycondensate, and (2) polyethyleneimine. The cationic polymer was found to be better than polyethyleneimine in removing phenol products. They concluded that those coagulants prevent peroxidase inactivation, and lead to the formation of larger precipitates. Ganjidoust *et al.* (1996) found that when using HRP and chitosan (coagulant) together, the overall removal of chlorophenols increased up to 80%, with the additional advantage that chitosan is highly biodegradable.

2.7. pH Dependence

The catalytic ability of the various types of peroxidases used for wastewater treatment (i.e. ARP, HRP, CiP, CMP, or SBP) differs. pH is an important factor, since there is a specific pH range where the catalytic ability of a peroxidase is optimal, thus larger turnovers are achieved reducing the quantity of enzyme required hence decreasing the treatment cost. Outside of that range, the catalytic ability can be considerably diminished or inhibited.

Due to solubility dependence on pH, the optimal pH is also dependent upon the compound being removed from the reaction mixture, as can be appreciated in Table 2-1. The optimal pH has been defined as the pH at which the greatest turnovers are achieved (Nicell *et al.*, 1993b).

An important issue to remark is that generally the range of pH in which peroxidases can catalyze the removal of phenols and aromatic compounds is wider than those for chemical treatment, this being another advantage over the conventional treatment. This is certainly an advantage since the pH from industrial wastewater can vary significantly even in the same industry.

In the case of ARP, Al-Kassim *et al.* (1993) reported good removal of eight different compounds in the pH range from 7.5 to 9.0. Nakayama and Amachi (1999) reported a maximum ARP activity at pH 5.0 to 8.0, depending upon the hydrogen donor used. In other studies, Al-Kassim *et al.* (1994) found an optimal pH range of 6 to 9 for HRP and CMP with a variety of phenolic compounds (refer to Table 2-1).

The use of ARP at pH values under 4 and over 10 is not recommended since its catalytic ability is very low (Ibrahim *et al.*, 1997b). The experiments to support this observation were carried out with 1 and 10 mM of phenol in dechlorinated tap water. Even though there is removal within this range, the optimal pH observed was 7.0 using

dechlorinated water and in the presence of PEG as additive, which may be shifting the optimal pH.

Since HRP has been widely studied by many researchers, different values for the optimal pH have been published. Wright (1995) mentioned that the discrepancies observed in the optimal values reported by various researchers (not only for HRP) may be due to the difference in the pH buffer species, pH buffer strength, initial concentration of phenolic compound, and the effect of soluble reaction products. He also noticed that the removal of phenolic compounds have demonstrated an absolute maximum at neutral to alkaline pH and in some cases a local maximum has been detected at an acidic pH, with low doses and incomplete removal.

Table 2-1. Optimal pH for various phenolic compounds in the presence of peroxidase.

	Al-Kassim *et al.* (1993)	Al-Kassim *et al.* (1994)		Klibanov (1982)	Nicell *et al.* (1993a)
Compound \ Enzyme	ARP	HRP	CMP	HRP	HRP
Phenol	[1]7.5 [2]8.1	8	9	3.5	8
o-cresol	8.6	-------	-------	4	-------
m-cresol	7.7	-------	-------	4	-------
p-cresol	7.8	-------	-------	5.5	-------
o-chlorophenol	7.6	-------	-------	7	-------
m-chlorophenol	8.6	-------	-------	7	-------
p-chlorophenol	7.6	-------	-------	5.5	-------
2,4-dichlorophenol	7.7	8	9	-------	8
2-chlorophenol	-------	8	9	-------	8
3-chlorophenol	-------	9	9-10	-------	9
4-chlorophenol	-------	9	9-10	-------	9
2-methylphenol	-------	-------	-------	-------	9
3-methylphenol	-------	-------	-------	-------	9
4-methylphenol	-------	7	8	-------	7

*The specific conditions under which these results were obtained may vary in enzyme concentration, buffer, H_2O_2 concentration or other parameters. For specific information about this data refer to the original sources. [1] Borate was used as buffer solution. [2] Phosphate buffer used in the reaction mixture.

Nicell *et al.* (1992, 1993b) observed that HRP exhibited catalytic ability between the pH of 4 and 10 for various phenolic compounds, but accomplished better removal within the pH range of 6 to 9. Negligible catalytic ability was reported below pH 2 and above pH 11. The optimum reported for phenol removal by HRP in the absence of PEG is 8.1.

Klibanov *et al.* (1983) reported precipitation of phenol using HRP in the range of pH 3 to 12, with the optimum at 9 using borate buffer.

Wu *et al.* (1993) detected good removal of phenol over the pH range 6 to 9 using HRP, with an optimum at pH 8.5 (using borate as buffer). As observed by Ibrahim *et al.* (1997a), the optimal pH shifted depending on the use of PEG or not. The borate buffer has shown to have protective effect from HRP inactivation. Because of this additional effect, a true optimal pH of 8.0 was selected. It is important to note that borate is not a suitable buffer solution for practical purposes because of its insecticidal action.

Similarly to other peroxidases, Soybean peroxidase (SBP) has also been found to have a wide action range of pH, with a maximum activity at pH 6.4, whereas in the range of pH 5.7 and 7.0 more than 90% of the activity rate was observed, and >10% of it was retained outside this range, but within the limits of pH 3 to 9. This shows that the SBP is slightly more sensitive to pH than HRP (Wright and Nicell, 1999), when compared with the results obtained for HRP by Nicell *et al.*, (1993b).

Ibrahim *et al.* (1997a, 1997b) found that in the presence of sodium phosphate as buffer, precipitate was formed after initiating the reaction, but with tap water no precipitate was produced, and the brown color of the mixture was not removed by filtration, consequently requiring alum to form floc and remove color.

Wright and Nicell (1999) noticed that the phenol removal observed in reaction mixtures containing either phosphate $\left(PO_4^{-3}\right)$ or orthophosphite $\left(PO_3^{-3}\right)$ was less than when using acetate, citrate or chloride. They suggest that this ion (phosphate) promotes

inactivation of SBP, becoming more significant at acidic pH. Thus, the use of phosphate or orthophosphite in acidic buffers (pH<5) should be avoided to prevent any inaccuracies of the results.

The use of coagulants (i.e. alum) in the reaction mixture to favor the color and by-products removal is an important factor to take into account when referring to pH. As it is well known, alum tends to decrease the pH in a liquid if its buffer capacity is low. Since the water has lower buffering capacity than any chemical buffer, the alum used in the experiments done by Ibrahim and coworkers (1997) shifted the initial optimum pH for those experimental conditions (pH = 7.0) to a lower value and decreased the removal efficiency.

Not only is the removal efficiency affected by the pH, but so are the kinetics of the transition from native enzyme to the various intermediates, as broadly studied by Dunford and Cotton (1976), and Critchlow and Dunford (1972) for HRP within the pH range from 2 to 11. The reaction rates observed were also distinct for each substrate, possibly because of a change in the different acid groups in the active site.

2.8. Stoichiometry

Based on the general mechanism of removal (refer to equation 2.4), it can be seen that for every mole of peroxide consumed, two moles of aromatic compound are oxidized, reflecting a peroxide/phenol ratio of consumption of 1:2 or 0.5, theoretically. Despite this, in practice the ratio of peroxide consumed to phenol removed from solution has been reported more frequently to be 1:1 (Nicell *et al.*, 1992, 1994; Al-Kassim *et al.*, 1994; Buchanan and Nicell, 1997). This general mechanism and 0.5 stoichiometry imply that the first formed dimers are not substrates of the enzyme and are readily precipitated from the solution (Wu *et al.*, 1999). As mentioned before, this is not always factual. If these dimers fail to precipitate, they still have the ability to return to the active site

forming higher molecular weight polymers, thus increasing the peroxide consumption and shifting the stoichiometry to a higher value.

The return of dimers into the active site increases the peroxide consumed as the enzyme oxidizes the dimers and larger intermediates until they precipitate from solution. The average size of precipitation polymer decreases with decreasing stoichiometry (Buchanan and Han, 2000). The 1:1 stoichiometry is based on the assumption that the chain of polymers formed would approach infinity before precipitation, where the limit of the peroxide/phenol stoichiometry would be 1.0.

However, Nicell *et al.* (1992) observed that for eight different phenolic compounds, the phenol removal was not linear with peroxide (except for 4-chlorophenol) but varying with the amount of peroxide supplied. As the concentration of peroxide was increased in the reactor, the ratio of peroxide consumed to phenol removed approached unity. As mentioned before, the ratio of consumption of peroxide to aromatic compound removal should approach unity as a limit.

Various examples of different and shifting stoichiometry have been reported in the literature. Al-Kassim *et al.* (1993) during their experimentation with ARP observed that the stoichiometry (peroxide/aromatic) varied from 2.0 to 1.2 as phenol concentration was increased from 0.5 to 1.0 mM. Al-Kassim *et al.* (1994) observed a 0.85:1 and 1:1 ratio of peroxide consumed to aromatic removed in the presence of excess CMP for 1 mM 4-chlorophenol and phenol initial concentration, respectively. Buchanan and Han (2000) observed that the stoichiometry shifted from 1.0 to 0.83 as initial phenol, peroxide and ARP concentrations were increased.

Dunford (1986) explained the reaction occurring between HRP and the dimers formed, since dimers are also HRP substrates, this increases peroxide requirements:

$$HRP + H_2O_2 \rightarrow HRP_i + H_2O \qquad (2.19)$$

$$HRP_i + AH_2 \rightarrow \cdot AH + HRP_{ii} \qquad (2.20)$$

$$\cdot AH \rightarrow \frac{1}{2}\ \text{dimmer} \qquad (2.21)$$

$$HRP_{ii} + \frac{1}{2}\text{dim}er \rightarrow HRP + product \qquad (2.22)$$

Besides the theory of the increasing peroxide consumption for the further reactions with dimers, other mechanisms have been proposed for the observed varying stoichiometry, involving the modification of the catalytic mechanism, but they have not been confirmed yet (Nicell *et al.*, 1994).

2.9. Temperature Dependence and Thermostability

Temperature stability and thermal inactivation are very important factors when analyzing the use of an enzyme in the treatment of industrial wastewater, since some industries have warm or even more elevated water temperature in their effluent stream.

As cited before, thermal inactivation due to denaturation of the sensitive tertiary protein structure will affect the active site, eliminating the catalytic ability of the enzyme.

In many reactions, as temperature is increased, the kinetics of the reaction increase, reducing the reaction time. In contrast with non-enzymatic reactions, the enzyme activity does not increase continuously as temperature is raised. The enzyme generally denatures when temperature is raised somewhat above that at which it is typically found (Bailey and Ollis, 1986). What happens is that the atoms in the enzyme molecule gain energy (thus greater tendency to move) as temperature is increased. When sufficient energy is acquired, the weak interactions holding the globular protein structure together are overcome, resulting on denaturation of the enzyme (Bailey and Ollis, 1986).

ARP has been shown to retain 100% of its activity when incubated for one hour at 5 °C or 25°C. 60% or more of its activity remained when it was incubated at 35 °C or 45 °C (for one hour), and at 55 °C, ARP retained only 30% of its total activity after one hour (Buchanan and Han, 2000). Based on these results, equation (2.23) was fitted for temperatures above 35 °C, showing a first order behavior, depicted as follows:

$$A_t = A_o \exp(-kt) \qquad (2.23)$$

where t = time in minutes, k = first order rate constant (min^{-1}), A_t = time dependent activity (U/mL) and A_o = initial activity (U/mL).

Wright (1995) found similar thermal dependence of SBP to that of ARP (equation 2.23), and ARP has shown thermostability similar to that of HRP, but considerably less than that of SBP (Buchanan and Han, 2000).

When stored at temperatures at, or below 25 °C, HRP is stable for long periods of time (Nicell, 1991). Based on this, Nicell *et al.* (1993b) proposed that the improvement in the catalytic lifetime at lower temperatures, compared to that at higher temperatures, can be a result not only of the thermal denaturing but also could be due to a slower rate of reaction. This implies that a lower concentration of free radicals in the solution would reduce the probability of a free radical bonding on the enzyme (based on the free-radical inactivation mechanism stated in previous sections).

Nicell *et al.* (1992) reported rapid inactivation of HRP for temperatures above 45°C. However, they observed that similar removal of 4-chlorophenol within the 5 to 65°C temperature range was possible by adding sufficient enzyme to overcome thermal denaturation, but the cost would increase significantly at elevated temperatures. The optimal range of temperature to have a significant improvement in catalytic efficiency should be below 35 °C.

Adding this extra cost to that due to inactivation by reaction products would markedly reduce the cost effectiveness of the process. For example, the average turnovers for HRP catalyzed phenol removal in the presence of PEG observed within 5 °C to 35 °C range are 9,300 compared with 4,100 at 65 °C. Thus, more than twice the HRP would be required to achieve the same removal (Nicell *et al.*, 1992).

At acidic and basic pH, thermal inactivation of HRP is more pronounced than at neutral pH, caused by an increase in protein denaturing at low pH and an increase in heme degradation at high pH. Thermal stability can be enhanced using various methods including optimization of the pH, the addition of calcium ions, the immobilization of the enzyme on a solid support, and the use of an organic solvent reaction medium (Wright, 1995). The effect of calcium addition against thermal inactivation can be related to the loss of calcium from the enzyme at elevated temperatures (Wright, 1995).

2.10. Kinetics

The kinetic constants observed for the general enzyme-catalyzed mechanism (equations 2.1, 2.2, and 2.3) have been determined by various researchers. Buchanan and Han (2000) reported a value of $2.3\times10^{6}\ M^{-1}s^{-1}$ for k_1, associated with the oxidation of the ARP by hydrogen peroxide. Abelskov *et al.* (1997) reported a kinetic constant k_1 for CiP of $(6.7\pm0.2)\times10^{6}\ M^{-1}s^{-1}$ using ABTS as substrate at six fixed concentrations ranging from 10 to 500 µM, and $(8.8\pm0.6)\times10^{6}\ M^{-1}s^{-1}$ for five fixed concentrations of feluric acid ranging from 10 to 100 µM, both performed at pH of 7.0, and varying the peroxide concentrations up to 1 mM. The kinetic constants k_2 and k_3 were also determined by Abelskov *et al.* (1997) based on the transient-state, over a broad pH range (5 to 10) with feluric acid as substrate, concluding that the constants are similar in magnitude, increasing the ratio k_2/k_3 from 1.0 at pH 5.0 to 10, at pH 10.0 (refer to Table 2-2).

Table 2-2*. Transient-state determination of kinetic constants k_2 and k_3.

pH	k_2	k_3	k_2/k_3
5.0	8.5 ± 0.2	8.9 ± 0.3	1
6.0	25 ± 1.2	22.2 ± 0.5	1
7.0	≥ 63	41 ± 4	1.5
8.0	≥ 120	48 ± 2	3
8.5	≥ 97	44 ± 2	2
9.0	≥ 73	18 ± 1	4
9.5	≥ 63	8.7 ± 0.2	7
10.0	38 ± 1	3.8 ± 0.05	10

*After Abelskov *et al.* (1997). k_2 and k_3 in $\mu M^{-1} s^{-1}$

Kjalke *et al.* (1992) observed that CiP, CMP and ARP exhibited identical values of the rate constants (within experimental error). However, the measured steady-state value of k_1 at optimal pH was different for the two substrates used, guaiacol and iodine. For the first, k_1 was $(3.8 \pm 0.2) \times 10^6\ M^{-1}s^{-1}$ at pH 7.5, and for the latter $(2.6 \pm 0.2) \times 10^6\ M^{-1}s^{-1}$ at pH 5.5. A k_3 value of $(2.5 \pm 0.1) \times 10^4\ M^{-1}s^{-1}$ at pH 5.0 and $(2.5 \pm 0.1) \times 10^5\ M^{-1}s^{-1}$ at pH 8.0 were observed with iodide and guaiacol, respectively (optimal pH for removal). This difference in the k_1 values observed could be due to iodine binding to native peroxidase (Kjalke *et al.*, 1992).

Critchlow and Dunford (1972) studied the values of k_3 at various pH values for *p*-cresol with HRP, observing that the value of k_3 diminished as pH increased .

Dunford and Cotton (1975) studied the behavior of k_2 and k_3 over a broad range of pH, using *p*-aminobenzonic acid as substrate and HRP, at 25 °C, observing that the values decreased as pH increased.

Job and Dunford (1976) also obtained these two latter rate constants for HRP using different phenols and anilines at pH 6.38 and 7.80. They reported a rate constant $k_2 = 2.76 \times 10^6\ M^{-1}s^{-1}$ for phenol. From the data obtained (data not shown) it is clear that the kinetic constants, k_2 and k_3, change depending upon the compound being reduced,

and the substituent effect on the benzene ring. This is also mentioned by Nicell (1994), who stated that the rate constants for the reduction of compound II and compound III are dependent on the particular aromatic substrate being oxidized.

These kinetic constants, as well as others, are affected by temperature, as reflected in the research on SBP done by Wright (1995), where the rate constant k_1 was observed to decrease slightly from $1.46\times10^7\ M^{-1}s^{-1}$ at 25 °C to $1.19\times10^7\ M^{-1}s^{-1}$ at 10 °C.

A compilation of the results obtained by various researchers when evaluating the kinetic constant k_1 at different pH and reducing substrates for ARP, determined at 25 °C, as well as k_2 and k_3 are shown on Table 2-3:

Table 2-3*. Kinetic parameters for ARP.

Kinetic parameter	pH	Substrate Used	Value ($M^{-1}s^{-1}\times10^{-6}$)	Source
k_1	n/a	None	7.1	Andersen *et al.* (1991)
	7	ABTS	6.7	Ablelskov *et al.* (1997)
	7	Feluric Acid	8.8	Ablelskov *et al.* (1997)
	7	None	9.9	Ablelskov *et al.* (1997)
	7	H_2O_2	2.3	Buchanan and Han (2000)
	7.5	Guaiacol	3.8	Kjalke *et al.* (1992)
	8.8	Luminol	2.7	Kim *et al.* (1991)
k_2	8.8	Luminol	24	Kim *et al.* (1991)
k_3	5	Iodine	0.025	Kjalke *et al.* (1992)
	8	Guaiacol	0.25	Kjalke *et al.* (1992)
	8.8	Luminol	2.2	Kim *et al.* (1991)

* After Han (1998).

3. MATERIALS AND METHODS

3.1. Materials

ARP (*Arthromyces ramosus* peroxidase; E.C. 1.11.1.7) with a purity of RZ (reinheitszahl) of 2.5, and catalase from bovine liver (E.C. 1.11.1.6) enzymes in dry solid form were purchased from Sigma-Aldrich Canada, Oakville, ON., and stored in the freezer at –15°C until needed. Stock concentrated solutions were prepared as needed by dissolving the solid enzyme in an aqueous solution at pH 7.4 using 0.1 M phosphate buffer. Stock enzyme solutions were stored at 4°C. Polyethylene glycol (PEG) with a molecular weight of 3,350 was purchased from Sigma Chemical Co., St. Louis, MO. The molecular weight was selected based on the results of Nakamoto and Machida (1992), who reported that PEG with an average molecular weight of less than 400 has little effect on protecting the enzyme against inactivation, and that with molecular weight above 1000 is most effective for enzyme protection. ACS grade solid phenol (MW 94.11, purity 99% or greater), ACS grade hydrogen peroxide (30% w/v), 98% 4-aminoantipyrine (4-AAP), ACS grade potassium ferricyanide, ACS grade sodium bicarbonate, and ACS grade monobasic and dibasic (anhydrous) sodium phosphate were purchased from Fisher Scientific Co., Edmonton, Alberta. Hydrogen peroxide was stored at 4°C, and diluted solutions were prepared using ultrapure water on the day of their use. Aqueous solutions of potassium ferricyanide and 4-aminoantipyrine for the different assays were prepared using ultra pure water and stored at 4°C until their use. Reaction solutions were buffered to pH 7.0 using a monobasic-dibasic sodium phosphate buffer prepared according to the method of Gomori (1955). A chromic-sulfuric acid mixture (cleaning solution) was purchased from Fisher Scientific Co., Edmonton, Alberta for the purpose of cleansing glassware.

3.2. Equipment

Colourimetric assays were monitored using a Pharmacia Biotech Ltd. Ultrospec 2000 UV/Visible spectrophotometer (wavelength range 190 to 1100 nm with 1 nm resolution) operated from an 80486 computer with the aid of the SWIFT II Applications Software, from Pharmacia Biotech. Full spectra scans (190 to 1100 nm) for enzyme inactivation studies were performed using a HP 8453 UV-Visible Spectrophotometer (wavelength range 190 to 1100 nm, with 1 nm resolution). Glass and quartz semi-micro cuvettes with a 1 cm optical path length and a 1.5 mL volume were obtained from Hellma (Canada) Ltd. In order to ensure the cuvettes have no residue of chemicals, all cuvettes were soaked in a chromic-sulfuric acid solution for a few minutes prior to each assay and rinsed with ultra pure water.

Reaction pH was measured using a Fisher Accumet pH meter 50. Three-point calibration (pH 4.00, 7.00 and 10.03) was performed with pH standard solutions purchased from Fisher Scientific Co., Edmonton, Alberta.

An International Clinical centrifuge manufactured by International Equipment Co. Neeham Hts., MA. was used to centrifuge samples at 3000 rpm for 30 minutes to separate formed precipitate before analyzing the samples.

Liquid volumes ranging from 20 µL to 10 mL were measured using Eppendorf micropipetters, Eppendorf Maxipettor and tips. Volumes from 10 mL to 1 L for reactants preparation were measured using Kimax and Pyrex volumetric flasks purchased form Fisher Scientific Co., Edmonton, Alberta.

An Elgastat Maxima HPLC unit, from Elga Ltd, Bucks, England, was used to produce ultra pure water, used during this research to prepare all solutions and for rinsing purposes.

A Mettler AE166 electronic balance from Mettler Instruments, Greifensce, Zurich, Switzerland (with a readability of 0.0001 g.), was used to weight the chemicals to prepare all the solutions.

3.3. Analytical Techniques

To determine the concentration of the various chemicals in solution, different colourimetric assays were used, as mentioned below. A detailed description of these assays, including procedures, theories and calculations can be found in Appendix A. All of the assays were performed in triplicate to ensure a maximum measurement error of 5%.

3.3.1. *Arthromyces ramosus* peroxidase Activity Assay

Enzyme activity was determined by a phenol/AAP colorimetric assay containing a solution of *Arthromyces ramosus* peroxidase, phenol, hydrogen peroxide (H_2O_2), and 4-aminoantipyrine (4-AAP) as color generating substrates. With excess amounts of phenol, H_2O_2, and 4-AAP, the initial rate of the reaction is directly proportional to the amount of active enzyme present in solution. The rate of color development of non-precipitating product, which absorbs light at a peak wavelength of 510 nm with an extinction coefficient of 6,280 M^{-1} cm^{-1} (based on hydrogen peroxide), was measured and converted to enzyme activity in the cuvette. This assay should be conducted under controlled conditions of substrate concentration, pH, and temperature due to the dependence of the activity measurements on these variables. One unit of activity (U) is defined as the number of micromoles of hydrogen peroxide converted per minute at pH 7.4 and 25°C in an assay mixture that contained the following in 0.05 M phosphate buffer pH 7.4 in a final volume of 1.0 mL: 10 mM phenol, 2.4 mM 4-AAP, 0.2 mM H_2O_2 and diluted enzyme sample. ARP concentrations are expressed as units of activity measured under standard assay conditions.

Since the molar concentration of active enzyme is directly proportional to the enzyme activity, a constant of proportionality, C_a, can be defined. During this research, two constants of proportionality were defined given that two different enzyme lots were used. For the first ARP stock used in this investigation, the average constant of proportionality was $C_a = 53.05 \times 10^{-9}\ M/(U/mL)$, where 1 nanomole of ARP (based on the heme absorbance at 403 nm and a peroxidase extinction coefficient, ε, of 102,000 M^{-1} cm^{-1}, (refer to Figure 3-1) corresponded to $18.9 \pm 1.1\,U$, and the second lot yielding an average constant of proportionality of $C_a = 66.7 \times 10^{-9}\ M/(U/mL)$, such that 1 nm of ARP equals $15.0 \pm 1.4\,U$.

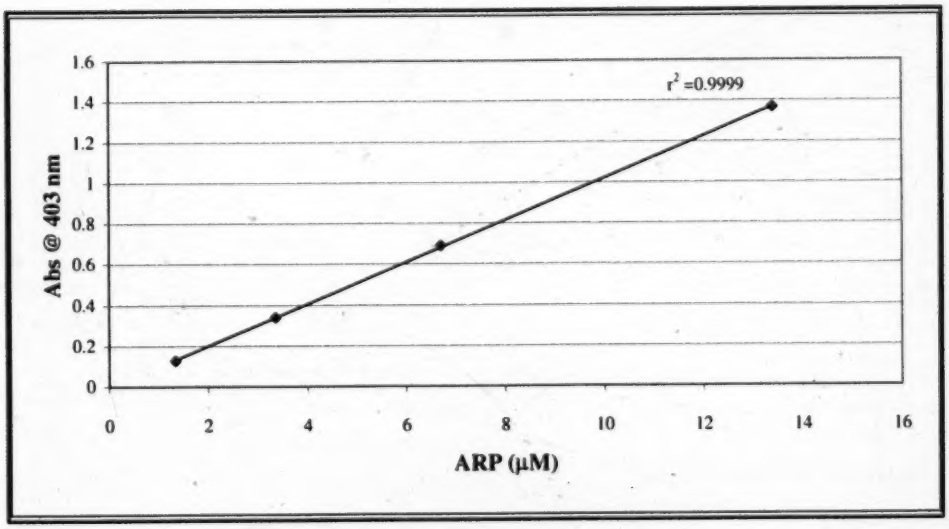

Figure 3-1. ARP extinction coefficient.

3.3.2. Total Phenol Assay

Phenol concentrations were measured using a colourimetric assay in which the phenolic compounds within a sample react with 2.08 mM 4-AAP in the presence of 8.34 mM potassium ferricyanide reagent. When the assay reaction is limited only by phenol, the extent of color development in the cuvette is directly proportional to the phenol concentration. After full development of the color, within 8 to 10 minutes, the absorbance at 510 nm was measured and converted to total phenol concentration in the cuvette (mM) using a calibration curve, resulting the following equation:

$$[phenol] = 0.0999 \times Abs_{510} \qquad (3.1)$$

where Abs_{510} is the measured absorbance at 510 nm and [*phenol*] is the phenol concentration in the cuvette, expressed in mM. For this assay it is important to note that the phenol concentration in the cuvette should not exceed 0.1 mM. Further calculations based on the dilution of the sample have to be taken into account (see Appendix A for detailed explanation). The calibration curve is presented in Figure 3-2.

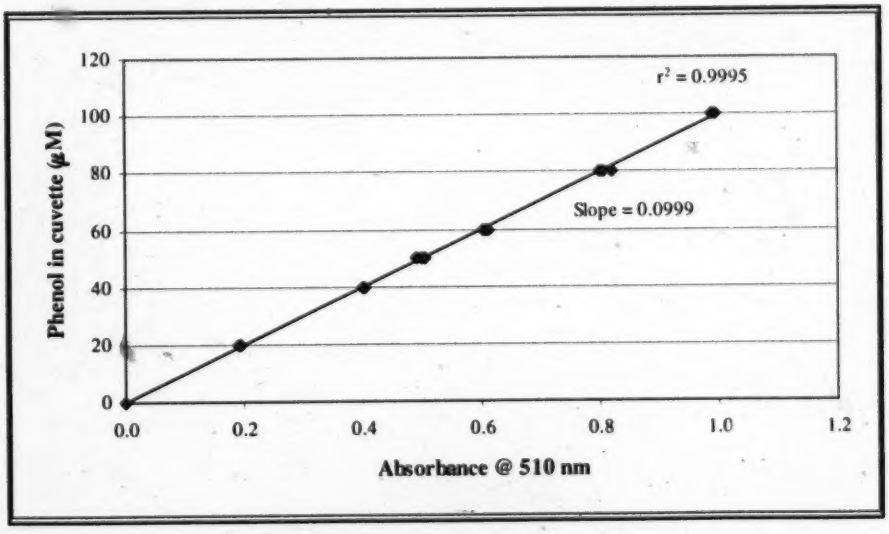

Figure 3-2. Phenol calibration curve.

3.3.3. Hydrogen Peroxide Assay

An end-point colourimetric assay was used to determine the concentration of H_2O_2 in the cuvette using ARP as catalyst, phenol, and 4-aminoantipyrine (4-AAP) as substrates at pH 7.4. For hydrogen peroxide to remain the limiting substrate, its concentration in the cuvette should not exceed 50 μM. After full color development, the absorbance at 510 nm was measured and converted to the hydrogen peroxide concentration (mM) in the cuvette by the following expression:

$$[H_2O_2] = 0.1592 \times Abs_{510} \qquad (3.2)$$

The result of hydrogen peroxide calibration is presented in Figure 3-3.

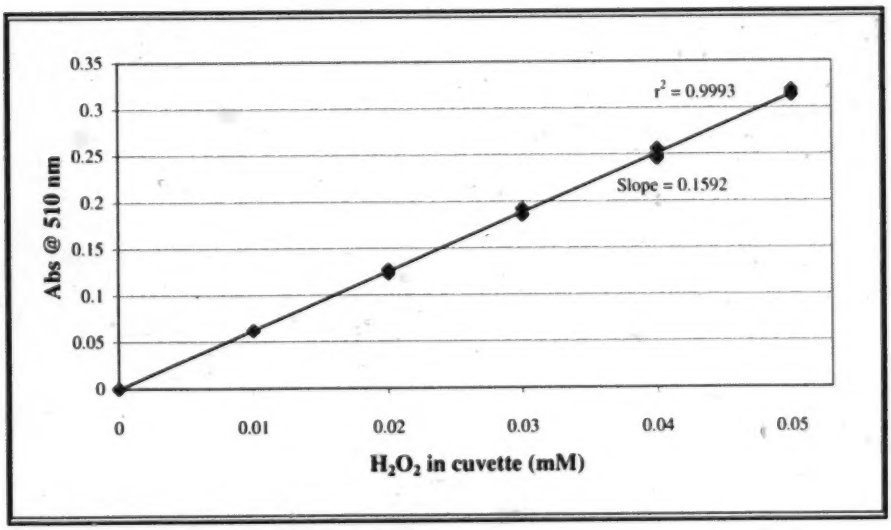

Figure 3-3. Hydrogen peroxide calibration curve.

3.4. Experimental Procedures

All the experiments were carried out in batch reactors with final volumes of 20 or 30 mL at room temperature (24 ± 0.5 °C). The reactants were prepared using a 0.1 M monobasic-dibasic phosphate buffer pH 7.0. Control samples were used in each set of assays to measure the actual concentrations of phenol and H_2O_2, and as reference to confirm that no phenol removal occurred in the presence of enzyme or H_2O_2 alone. All assays were performed in triplicate using the same stock reactants.

3.4.1. Batch Reactor Operation

Batch reactions used to determine reaction stoichiometry were conducted in 30 mL capped vials, with a final volume of the reaction mixture of 20 mL. The mixture consisted of 400 mg/L PEG to provide maximum enzyme protection, phenol, excess ARP to ensure that the removal of phenol would not be limited by enzyme, and a highly concentrated H_2O_2 solution (various concentrations) to minimize volume changes. The reactions were initiated by adding ARP to the solution containing the phenol, PEG and H_2O_2, and the reactants were mixed continuously throughout the 24 hour test with a Teflon-coated micro stir-bar and a magnetic stirrer. After the reaction was initiated, the mixture turned brown and precipitate was formed gradually.

After the reacting period, 5 mL samples were withdrawn from the batch reactors and centrifuged at 3000 rpm for 30 minutes to precipitate the product formed. The supernatant was then diluted to meet with the maximum concentration limits on the assay (as mentioned in subsection 3.3.2), and the phenol remaining in the cuvette was measured as described before.

To ensure that the enzyme concentration used during these tests (7 U/mL) was not limiting the removal of phenol, more enzyme was added after the reaction period, and no

further removal of it was noted. Thus, the ARP concentration used was enough to remove the highest phenol concentration used during this investigation (8 mM).

3.4.2. Step Addition of Hydrogen Peroxide

30 mL batch reactors were used to study the effect of step H_2O_2 addition on the removal of phenol, in a mixture containing 400 mg/L PEG, 4 mM phenol, limited enzyme (46.5 nM ARP) to restrict the phenol removal to less than 100% so that any enhanced removal could be observed. Two sets of experiments were carried out, each one with 4 settings, according to the peroxide used on each: (1) control, with no enzyme; (2) 4 mM H_2O_2 as a single dose; (3) 4 mM H_2O_2 as two equal doses (2 mM); and (4) 4 mM H_2O_2 as four equal doses (1 mM). The first set of batch tests was allowed to react overnight (24 hrs) after the addition (if applicable) of the next dose of peroxide. The second tests were conducted on a time limiting basis, restricting the reaction time to one hour before the next dose of peroxide was added. Before each addition of peroxide, a 3 mL sample was withdrawn from each reactor and poured into a glass tube containing 250 µL of a concentrated solution of catalase (2.5 g/L) to immediately stop the reaction by consuming any remaining peroxide. The samples were centrifuged for 30 minutes, and the supernatant analyzed for phenol, as described before.

Catalase is an antioxidant which decomposes hydrogen peroxide to water and oxygen by the following pathways:

$$ferricatalse + H_2O_2 \xrightarrow{k1} compound\ I + H_2O \qquad (3.3)$$

and

$$compound\ I + H_2O_2 \xrightarrow{k2} ferricatalase + H_2O + O_2 \qquad (3.4)$$

3.4.3. Spectrophotometric Measurements (Enzyme Inactivation)

Spectral changes of ARP upon addition of hydrogen peroxide were monitored within the range of 190 to 1100 nm. These experiments were carried out in 1.5 mL quartz cuvettes with a final volume of 1 mL, containing ARP, sodium phosphate buffer pH 7.0 and peroxide in different concentrations. An initial spectral scan of the native enzyme was taken prior to H_2O_2 addition as a reference. After the addition of peroxide, the cuvette was capped and inverted 3 times, and the time was recorded. Frequent spectral scans were performed at different times (up to 24 hours) to track the changes occurring to the enzyme. The cuvettes were covered with a plastic film to avoid evaporation of the solution mixture, causing errors on the readings.

4. RESULTS AND DISCUSSION

4.1. Stoichiometry

According to the overall reaction for aromatic removal using peroxidase as a catalytic agent (equation 2.4) for each mole of hydrogen peroxide consumed, two aromatic substrate molecules are oxidized and released as free radicals into the solution, yielding a 1:2 peroxide to aromatic ratio. This stoichiometry suggests that the formed free radicals couple forming dimers, and those dimers are not substrates for the enzyme and are readily precipitated from the solution (Wu *et al.*, 1999). Nevertheless, this theoretical ratio of 0.5 has never been observed in practice in any of the different peroxidases studied (e.g. ARP, HRP, CiP, CMP, SBP). Conversely to that prediction, a number of different consumption ratios have been reported in the literature by many researchers (Buchanan and Han, 2000; Cooper and Nicell, 1996; Nicell, 1994; Nicell *et al.*, 1992, 1994; Al-Kassim *et al.*, 1993, 1994).

Nicell (1994) explained that the inconsistency between the theoretical and measured stoichiometry is caused by polymers that fail to precipitate from the solution, which can react again forming higher polymers, therefore consuming more peroxide and increasing the stoichiometry value. Yu *et al.* (1994) found that some of these coupling products, besides being substrates for the enzyme, react faster than the parent phenol (e.g. *p,p'*-biphenol, *o,o'*-biphenol), supporting the latter explanation by Nicell. Dunford (1976) suggested the path followed by the reacting dimers (refer to equations 2.17 to 2.20 in subsection 2.8).

However, the ratio of consumption has been reported more frequently to be 1:1 (Nicell *et al.*, 1992, 1994; Al-Kassim *et al.*, 1994; Buchanan and Nicell, 1997), or tending towards it as a limit, assuming that the chain of polymers formed would approach infinity before precipitation. Stoichiometry seems to vary (increasingly) with the initial concentration of peroxide provided, as observed by Nicell *et al.* (1992) with eight

different compounds, where the ratio of peroxide to phenol approached unity as the concentration of peroxide was increased in the reactor.

Due to these varying values reported, the stoichiometry between initial hydrogen peroxide and phenol removed from solution was studied in an attempt to model it based either on initial phenol, peroxide, or enzyme supplied.

Four series of batch tests were conducted at room temperature (24.0 ± 0.5 °C) to study and possibly predict the relationship between these parameters. The tests consisted of 1.04, 2.15, 4.40, and 8.03 mM phenol solution mixed with 400 mg/L of PEG (both in sodium phosphate buffer pH 7.0), peroxide concentrations ranging from 0 to 8.4 mM, and excess dose (7 U/mL - 371.4 nM) of ARP was used to ensure that the removal was only limited by H_2O_2 (refer to Appendix B.1 for complete data). To corroborate that the enzyme did not limit the removal, ancillary peroxidase was added to the reaction, confirming that further removal was statistically insignificant (data not shown). The mixture was allowed to react for 24 hours to ensure completion of the reaction.

The attempts to model stoichiometry as a function of initial concentrations of the diverse constituents were unsuccessful. Nevertheless, as observed by Nicell and colleagues (1992), as the initial ratio of peroxide to phenol was increased, the stoichiometry, in all cases, tended to increase towards unity (refer to Appendix B.1). Apparently, there is not a defined relationship between the initial phenol and peroxide concentrations and stoichiometry. This is in agreement of the results obtained by Han (1998).

Relationships between moles of hydrogen peroxide consumed per moles of phenol removed were fitted with linear regression (refer to Figure 4-1 to Figure 4-4, average values shown), which represents the average stoichiometry observed for the tests conducted.

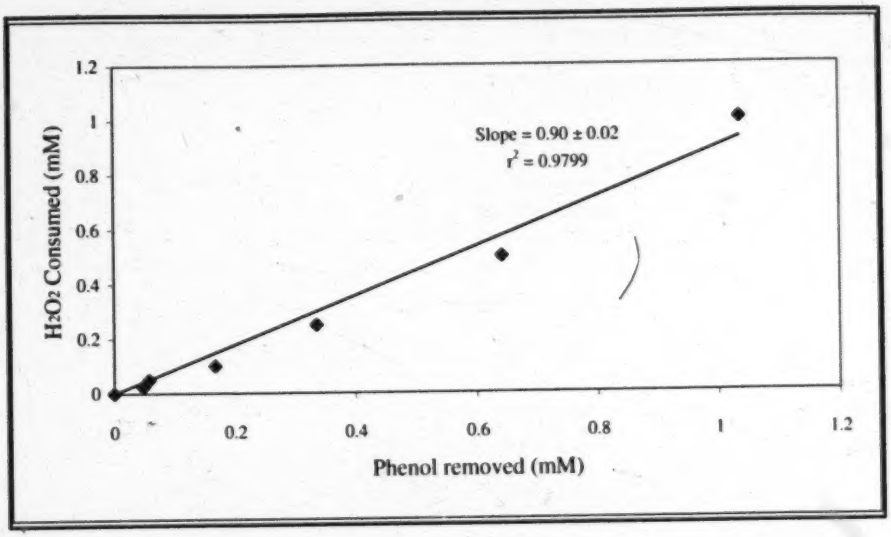

Figure 4-1. Stoichiometry between consumed H_2O_2 and phenol removed (1.04 mM initial phenol).

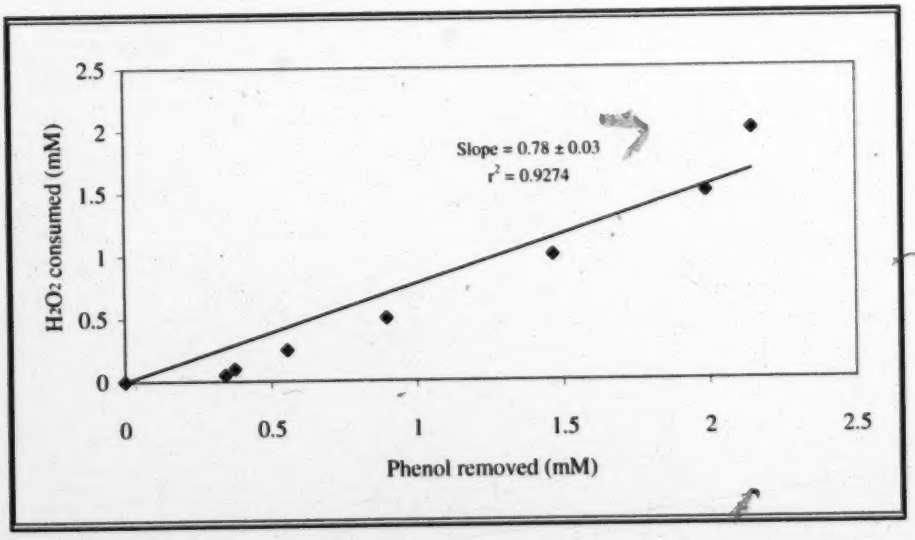

Figure 4-2. Stoichiometry between consumed H_2O_2 and phenol removed (2.16 mM initial phenol).

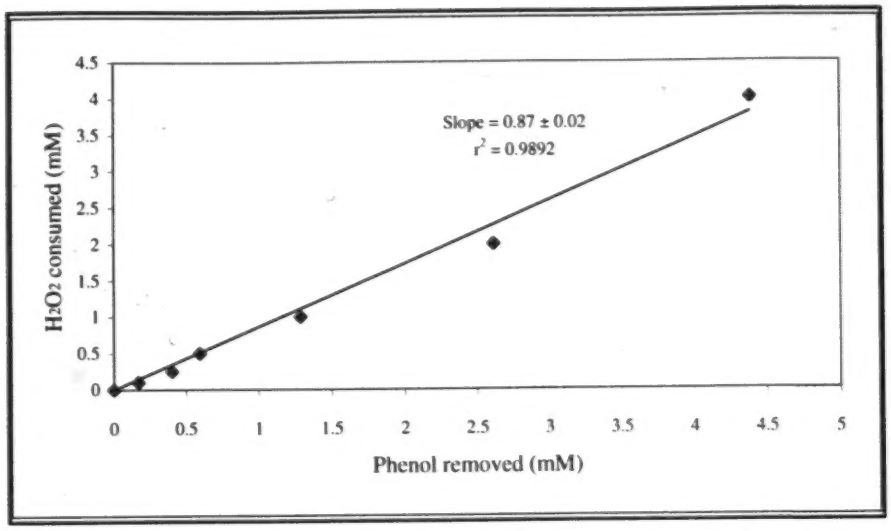

Figure 4-3. Stoichiometry between consumed H_2O_2 and phenol removed (4.40 mM initial phenol).

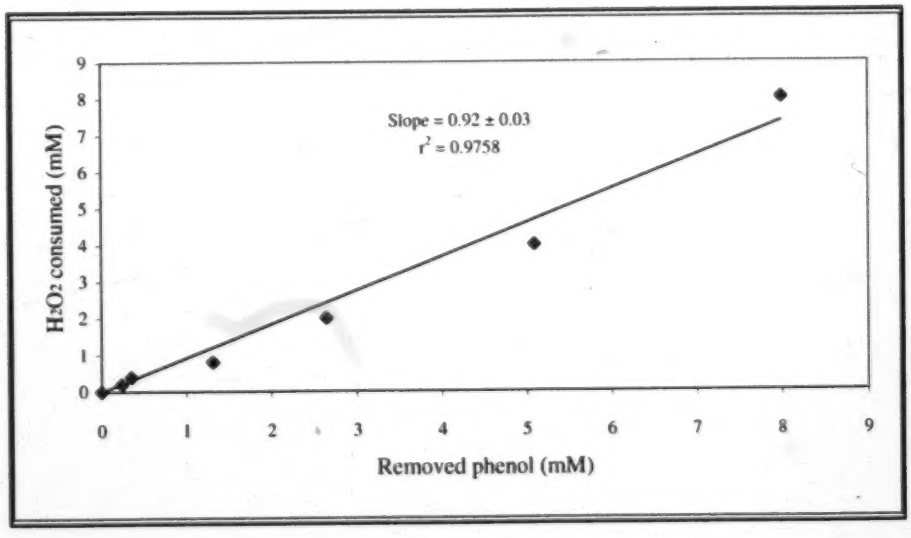

Figure 4-4. Stoichiometry between consumed H_2O_2 and phenol removed (8.03 mM initial phenol).

The average stoichiometry, represented by the value of the slope of the line, showed an increasing value (with the exception of the test using 1.04 mM initial phenol) from 0.78 to 0.92 as initial phenol was increased.

A possible explanation for the inconsistency observed in stoichiometry may be attributed to the random formation of a mixture of soluble and insoluble products, even though some of them are most likely coupling (Yu *et* al, 1994), with different coupling configurations as depicted in Figure 4-5b. This would affect the propensity of each one of them to couple and even return to the active site of the enzyme for further formation of higher oligomers, such as dimers, trimers, tetramers, etc. (refer to Figure 4-5a). This tendency, jointly with the fact that larger polymers formed would reflect an increase on peroxide consumption (Nicell, 1991), and hence the observed stoichiometry (i.e. H_2O_2/AH_2) would vary in a semi-arbitrary path. Further experiments are required to identify which is the most likely coupling configuration for phenol with ARP, if any, and ARP affinity for these formed dimers to react again with the enzyme.

A second experiment was performed in order to analyze the dependence of phenol removal and hydrogen peroxide consumption based on enzyme concentration. For this test, batch reactors provided with a fixed amount of phenol (4.22 mM) and peroxide (3.71 mM), were allowed to react with different enzyme concentrations ranging from 0 to 7 U/mL (0 to 371.4 nM) in the presence of 400 mg/L PEG. The phenol remaining and peroxide consumed were measured after a 24-hour reaction period.

As it can be appreciated in Figure 4-6 (average data shown from three replicates performed, refer to Appendix B.2 for complete data), an initial rate of consumption of phenol behaved linearly up to approximately 0.9 U/mL (47.7 nM) of enzyme supplied. This suggests that the phenol removal increased linearly (refer to Figure 4-7) with increasing enzyme dose until the amount of phenol became limiting, indicating that the number or catalyzed reactions per enzyme molecule (i.e. turnovers) are independent of

the enzyme dose. Ibrahim *et al.* (1997b) obtained similar results with a linear phenol removal (for 1 and 10 mM) until phenol was depleted from the reactor.

Since the phenol removed showed a linear relationship, turnovers should behave similarly. As it can be appreciated in Figure 4-6, turnovers remained fairly constant over the range of 0.3 to 1 U/mL of enzyme supplied, confirming the before mentioned linear relationship between phenol and ARP. A sudden drop on turnovers was observed at, and after 2 U/mL ARP. There are three possible explanations for this response: (1) at 2 U/mL of enzyme, all the phenol was depleted from the reaction mixture, thus the number of reactions completed per mole of enzyme decreased, since more enzyme was present and no substrate was available, decreasing the aromatic/enzyme ratio, hence turnovers; (2) at larger enzyme quantities, the available amount of enzyme for inactivation is raised, thus, the probability of a free radical hindering the access to the active site, consequently blocking the entrance of substrate for further catalytic reactions, as mentioned by Nicell and coworkers (1992, 1993b); and (3) as the quantity of enzyme present in the reaction mixture is raised, more enzyme is inactivated by hydrogen peroxide, with the consequent formation compound III and other possible inactive forms of the enzyme. Obviously, this could be the combined effect of the three systems.

Based on the observed turnovers achieved by different enzyme concentrations, it can be concluded that, for ARP, step addition of it would not markedly improve the removal of aromatic compounds. This can be regarded when a comparison in the turnovers for the lowest enzyme concentration (0.3 U/mL), and for complete removal of phenol (~1 U/mL), is made. Both enzyme concentrations yielded similar number of catalytic cycles.

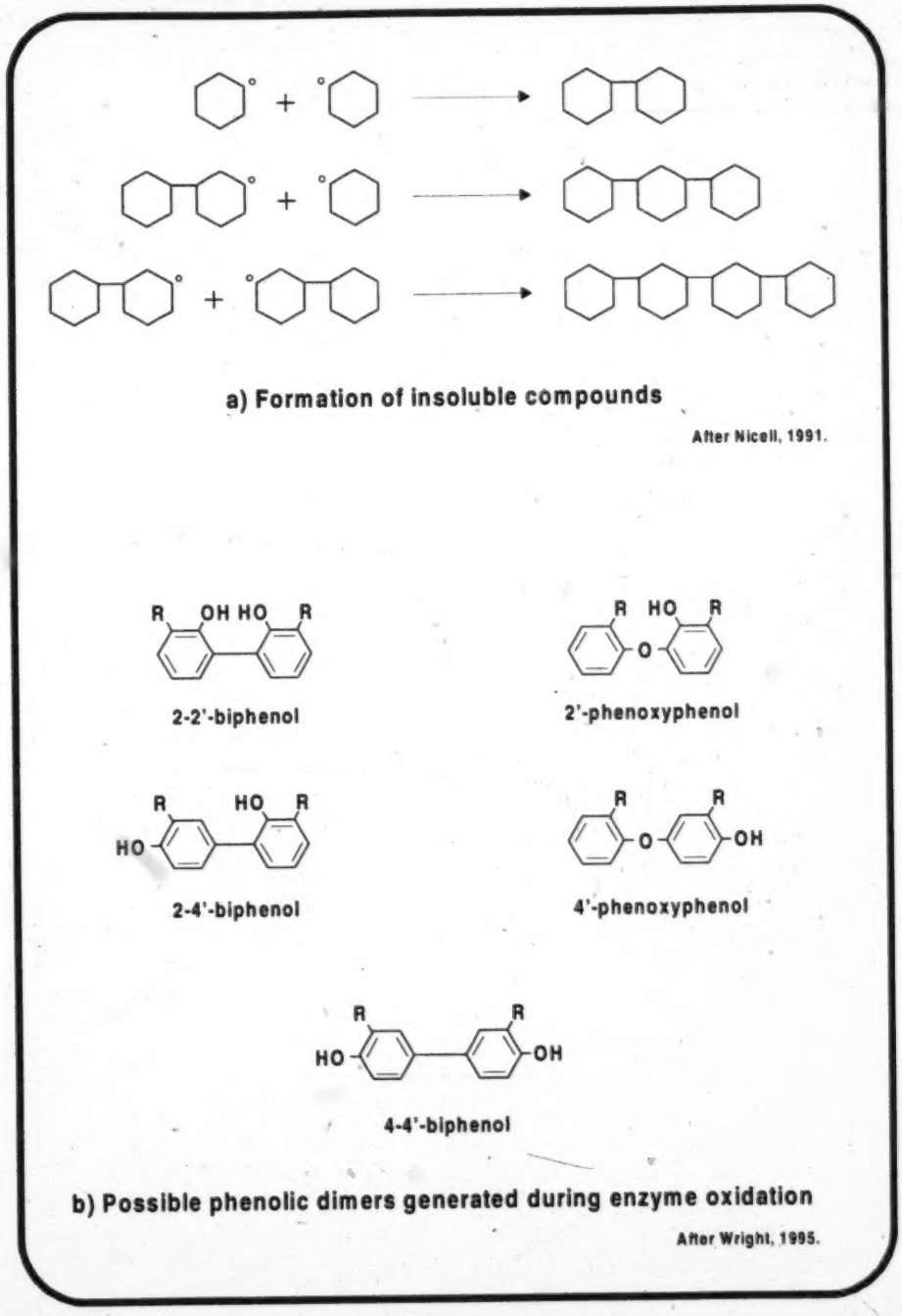

Figure 4-5. Reaction of free radicals to form insoluble polymers and possible dimmer combination of observed with phenol.

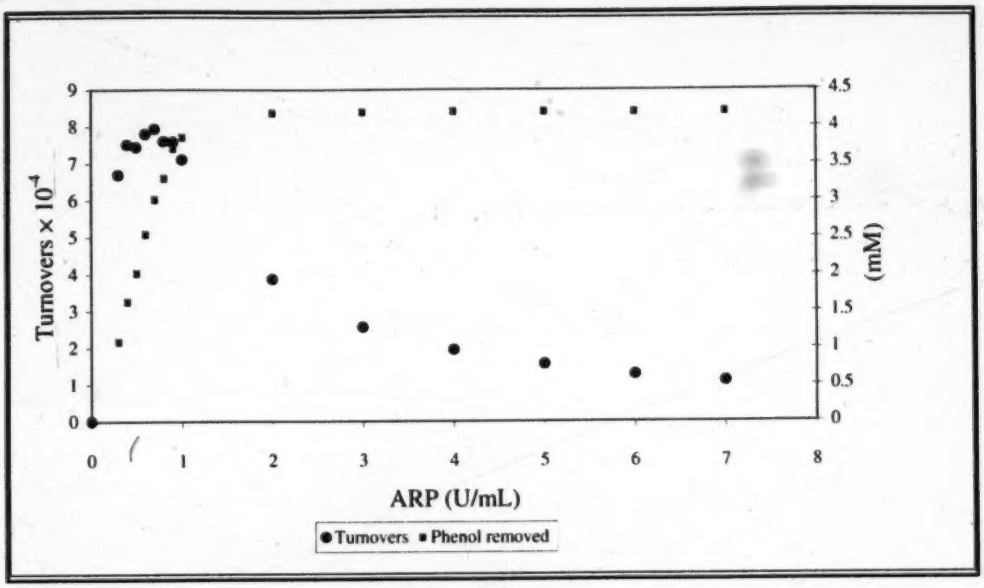

Figure 4-6. Removal of phenol and turnovers observed with different enzyme concentrations.

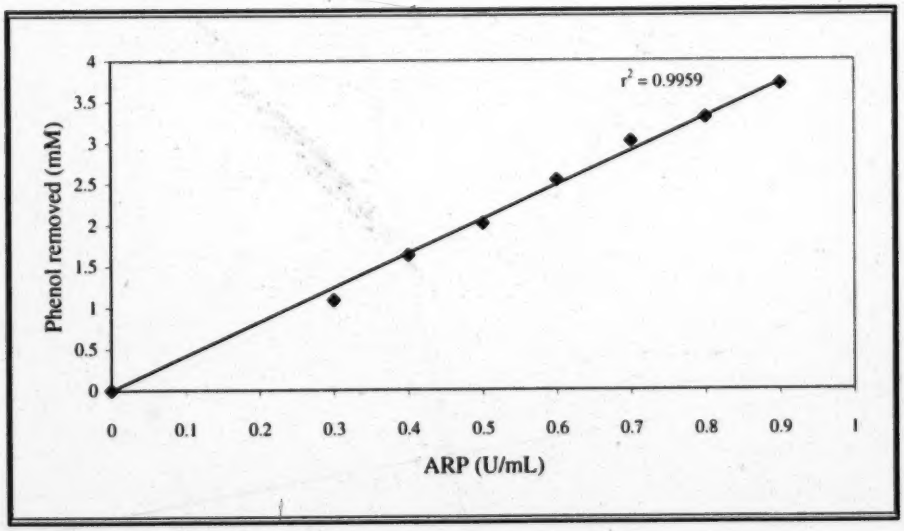

Figure 4-7. Linear removal of phenol with increasing enzyme dose.

These observations also lead to conclude that the mechanism of inactivation for ARP is more severe due to peroxide-related inactivation (i.e. compound III formation) and/or ARP entrapment. Otherwise, the enzyme step addition would increase the aromatic removal, as observed by Nicell *et al.*, (1992, 1993b), Al-Kassim *et al.* (1994), and Klibanov *et al.* (1983). Thus, step addition would only increase the hydraulic retention time for phenol removal, increasing the operation costs, which is not a suitable option for large-scale applications.

Based on the observation concerning the rate of consumption observed for H_2O_2 (refer to Figure 4-8) it can be deduced that the utilization of peroxide was most likely in the normal peroxidase cycle (equations 2.1 to 2.3) because of the close to linear relationship between enzyme dose and peroxide consumption. Buchanan and Nicell (1997) reported values of k_3 $(E_{ii} \rightarrow E)$ and k_{app} $(E_{ii} \rightarrow E_{iii})$ of 2.47×10^5 and 13.5 (average from values reported), respectively, which differ by four orders of magnitude from each other. This observation also supports the concept mentioned by Aitken and Heck (1998), where the loss of enzyme activity by inactivation processes would be significantly lower (5 to 6 orders of magnitude) in the presence of a reducing substrate.

Even though the rate of the reaction between compound II with aromatic substrate is much faster than that of hydrogen peroxide with compound II, an increased tendency in hydrogen peroxide consumption can be observed. This observation leads to one of the following explanations: (1) peroxide is being consumed by larger polymer chain formation; (2) as more phenol is being removed, more H_2O_2 is being consumed; or (3) peroxide-related inactivation is occurring. The first possibility can be discarded since a higher instantaneous free radical concentration, provoked by the increased enzyme dose, would favor the formation of polymers with smaller average sizes, which, according to Nicell (1991), would lower the quantity of peroxide consumed. The phenol removal showed a linear relationship with the amount of enzyme supplied, with no tendency to increase (further than the linear relationship) as enzyme did, discarding the second option.

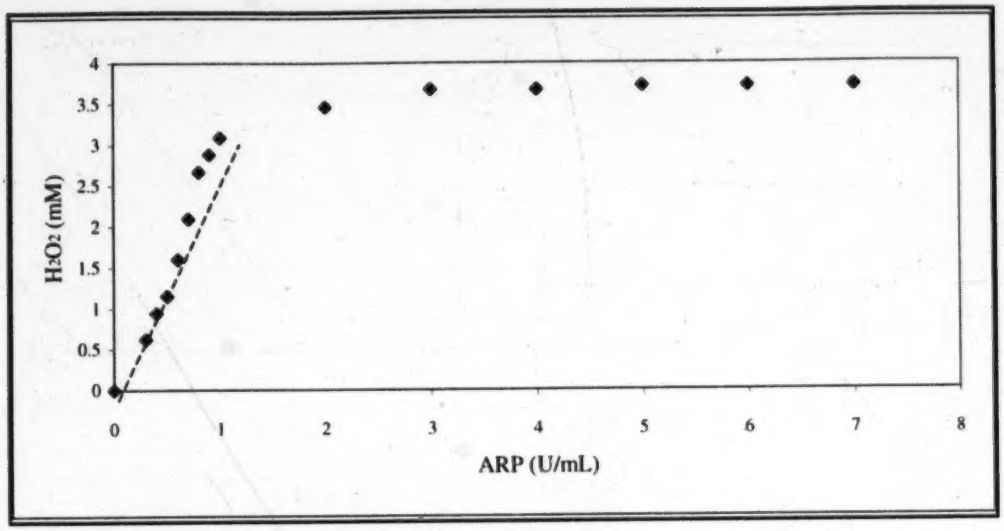

Figure 4-8. Consumption of hydrogen peroxide in the reaction.

So this suggests that as the enzyme concentration was raised, the increasing tendency of hydrogen peroxide consumption was due to the creation of inactive forms of the enzyme, which even at a slow formation rate influenced enzyme inactivation and peroxide consumption, this in agreement to the previous discussion of turnovers achieved. The effect on stoichiometry of increasing ARP dose is shown in Figure 4-9.

As can be observed in Figure 4-1 to Figure 4-4, linear regression did not provide a truly representative fit to the data obtained during this research, but a curve would better characterize the true trend. However, the results were plotted in such way in order to provide a more faithful comparison of the stoichiometric values here obtained, with those from other researches (Nicell *et al.*, 1992, 1994; Al-Kassim *et al.*, 1993, 1994; Buchanan and Nicell, 1997; Buchanan and Han, 2000). Linear fit has been mainly used to provide an average stoichiometry with the use of HRP because of the strong linearity observed for that enzyme.

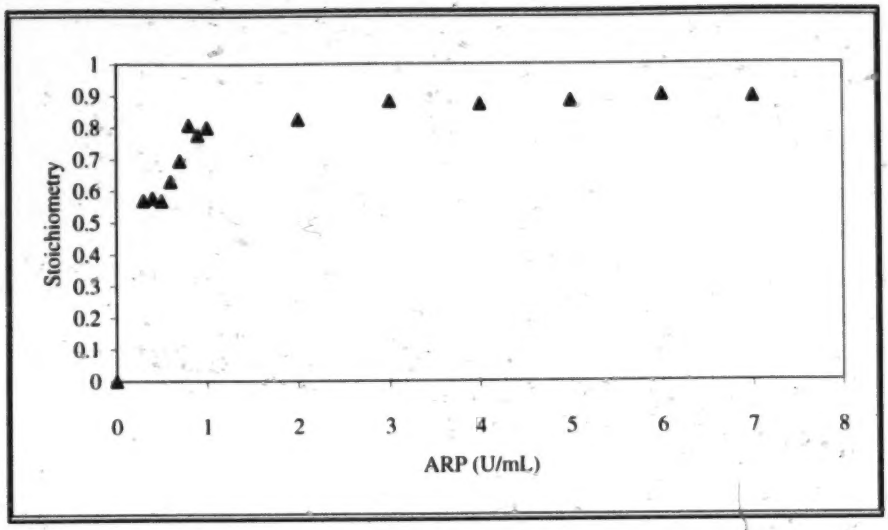

Figure 4-9. Stoichiometry observed between moles of peroxide consumed and phenol removed.

Furthermore, it is distinguishable in Figures 4–1 to 4-4 that the data values have a tendency to increase (reflecting an increase in stoichiometry) as the initial concentration of hydrogen peroxide (that was directly correlated to the peroxide consumed) was increased. Likewise, an increase of peroxide consumption was observed with the second set of experiments, as previously elucidated (Figure 4-8), which was reflected in an increase of stoichiometry as the initial ARP dose was increased (Figure 4-9). The preceding observations suggest that as more peroxide, or the quantity of enzyme is in excess, there is a higher tendency in hydrogen peroxide consumption. Nevertheless, this higher peroxide consumption was not reflected in a better phenol removal, which is an indication that the increasing peroxide consumption was not exerted in the regular peroxidase cycle, but most likely in interactions directly with the enzyme in the formation of inactive compounds (e.g. compound III).

4.2. Step Addition of Hydrogen Peroxide

Based on the previous observations, and since a step addition of enzyme would not increase the removal efficiency markedly, but probably increase enzyme inactivation due to free radicals and mostly hydrogen peroxide mechanisms, two set of tests were performed to evaluate the phenol removal performance on a step addition of hydrogen peroxide.

The two tests were supplied with 4.2 and 3.65 mM phenol, 400 mg/L PEG, limited enzyme (0.78 U/mL) to control the phenol removal in order to observe any improvement in its removal, and peroxide (4 mM), which was added in three different ways: (1) the 4 mM H_2O_2 were added as a single dose; (2) peroxide was added in two separate doses, each of 2 mM; and (3) peroxidase was added at four different times, each dose of 1 mM. The phenol removal was then determined after the specified reaction time (1 hour for the time limiting tests, and 24 hours for the non-time limiting). The results of these experiments are shown in Figure 4-10 and Figure 4-11 (average values shown, refer to Appendix B.3 for complete data. The arrows represent the time when peroxide was added.

In Figure 4-10, when the experiment was performed under time limiting conditions, the phenol removal was similar in the three different modes of peroxide addition. However, when comparing these results with those when the reaction mixture was allowed to react for a 24 hours period (refer to Figure 4-11), it can be appreciated that the removal efficiency increased slightly when the peroxide was added as four different doses. Note that the difference in removal (error bars) is statistically significant (statistical data not shown).

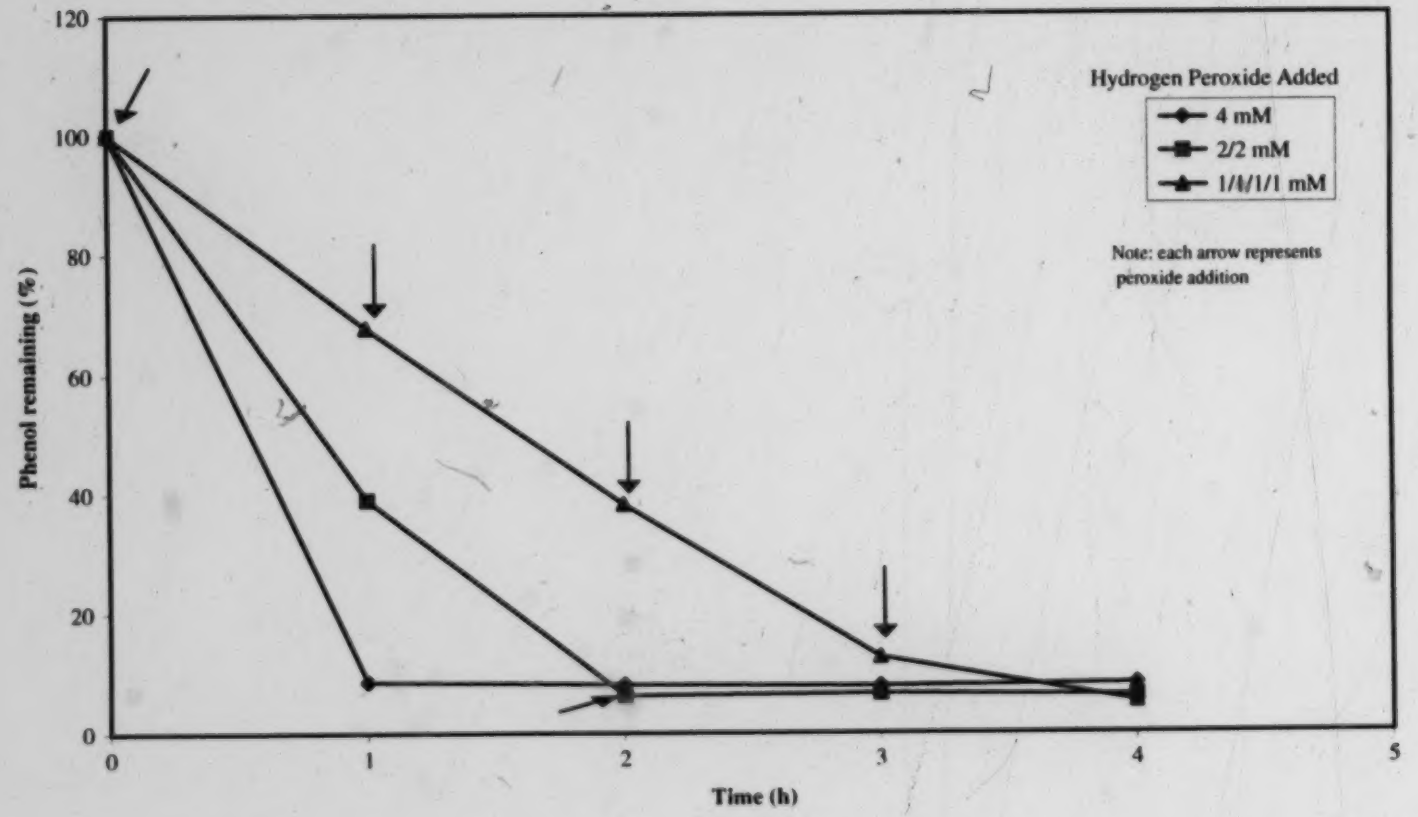

Figure 4-10. Phenol removal based on step addition of hydrogen peroxide on a time limiting basis.

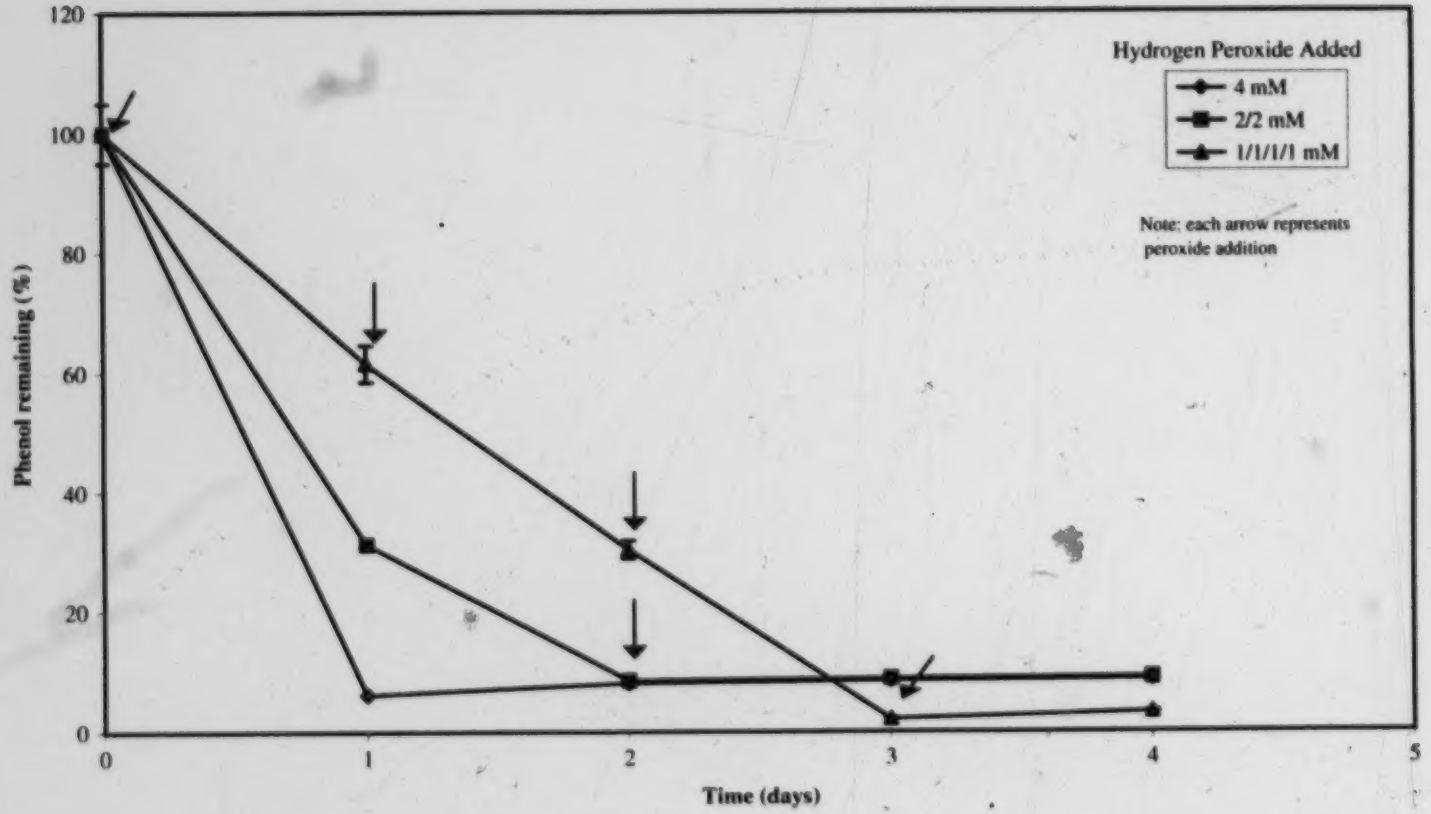

Figure 4-11. Phenol removal based on step addition of hydrogen peroxide.

Based on these results, the theory of free radicals inactivation as a dominant mechanism would be dubious, since comparable phenol removal was achieved when a higher concentration of instantaneous free radicals was present, after the 4 mM H_2O_2 were added at once, and also with the extended addition of peroxide in four doses of 1 mM H_2O_2, where the instantaneous concentration would be lower, thus better removal achievement would be expected. Thus, either the free radicals do not have an important effect on enzyme inactivation, or there is no such mechanism of inactivation. Two possible explanations are mentioned in literature: (1) as observed by Buchanan and Nicell (1998), the rate constant for inactivation of HRP by free radicals was zero in the presence of PEG, also used during these experiments; and (2) the latter statement is in agreement with Nakamoto and Machida (1992), whom declare that if the phenoxy radicals inactivation mechanism proposed by Klibanov *et al.* (1983) would be the case, it would be difficult or impossible to reduce its inactivation.

On the other hand, when the reaction was allowed to react for a longer period of time, an improvement on phenol removal was observed. This can be explained as follows: when the reaction time was limited to 1 hour, the inactive form(s) of the enzyme produced with peroxide (i.e. compound III or probably E_i-H_2O_2) were not allowed to return to the native enzyme form, due to its slow returning time, thus reducing the removal efficiency (at least during the observation time). But when enough reaction time was available, at least some of those inactive forms of the enzyme returned back to the native state, hence catalyzing more phenol and removing it from the solution.

4.3. Spectrophotometric Measurements (Enzyme Suicide Inactivation)

As discussed in section 2.4, some side reactions in the catalytic enzyme cycle include the formation of compound III, a non-permanent catalytic inactive form of the enzyme. The possible pathway to the formation of compound III, other intermediates and P-670 were described by Arnao *et al.*, (1990b), and depicted in equations (2.11) to (2.14).

Arnao *et al.* (1990a), predicted the formation of compound III in the absence of aromatic substrate explaining the pathway followed by compound I to yield compound II, which is required before compound III can be produced (equations 2.11 and 2.14). An explanation of this behavior is that the HRP/peroxide system acts as a catalase system, where H_2O_2 acts either as an oxidant or as a reductant (Arnao *et al.*, 1990b). So, after the formation of compound I, its reduction can be carried out by: (1) a two-electron transfer, yielding oxygen and native enzyme, and (2) a one-electron transfer forming compound II and a superoxide anion (refer to Figure 2-3), which seems to be a minor pathway compared with the catalase reaction (Arnao *et al.*, 1990b). From the intermediate of compound I and peroxide, a compound called verdohaemoprotein, also referred as P-670 because its characteristic peak absorbance at a wavelength of 670 nm, is formed (refer to equation 2.11). This P-670 intermediate is a permanently inactive form of the enzyme.

Thus, a study on the behavior of ARP with peroxide in the absence of reducing substrate was performed. Spectral changes were monitored at different times (up to 24 hours) in the UV/visible range from 190 to 1100 nm for two different batch tests, containing concentrated enzyme (15,531 nM) and either 0.25 or 4 mM H_2O_2.

The general behavior, independent of the initial peroxide concentration, as predicted, showed that immediately after the addition of hydrogen peroxide (7 seconds in both cases) the native enzyme, with the largest absorbance peak at a wavelength at 405 nm, shifted to the characteristic spectra reported for compound III of ARP, with the largest peak at 418 nm, and two smaller peaks at 548 and 582 nm (refer to Figure 4-12)[2]. This seems to be the representative absorbance of compound III of ARP, since it was also observed by Han (1998). Subsequently, a slow decrease in the absorbance of compound III representative peaks took place in the following minutes, until the peaks at 548 and 582 nm disappeared completely within 20 minutes (depending on the initial peroxide concentration, as will be discussed later), and the largest peak slowly shifted back from

[2] Refer to Appendix B.4 for complete spectra of Figures 4-12 and 4-14.

418 to 405 nm. However, at that point in time, the absorbance at 405 nm was lower than that observed initially, but it recovered slowly to certain extent with time.

With the observed spectra of compound III, it can be concluded, as observed by Andersen *et al.*, (1991), that CiP is virtually identical to ARP, showing characteristic spectra of compound III at 405, 547, 582, and a fourth peak (not observed with ARP) at 649 nm.

A detailed look of the two different tests revealed some important information regarding inactive forms of the enzyme. Comparing the initial and final spectra of the enzyme with 0.25 and 4.0 mM H_2O_2 concentrations, (refer to Figure 4-13 and Figure 4-14, respectively), the time and degree of reversion from compound III to native enzyme was dependent on the initial quantity of peroxide provided (consider that the same H_2O_2 volume was used to avoid differences due to dilution). When 0.25 mM of peroxide was used, the enzyme recovered its initial activity (within experimental error), but with 4 mM, the final absorbance was noticeably lower. Note that the remaining H_2O_2 after the 24-hour reaction period was zero in both cases, indicating that no more H_2O_2 was present in the reaction mixture provoking more compound III formation. The rate of return to the native form was also influenced by the initial quantity of peroxide provided: it took 3 minutes to start returning from compound III to native enzyme for the sample containing 0.25 mM, and 50 minutes for that with 4 mM H_2O_2. The reason for the increased delay observed is that more cycles (hence more time) are required to convert all the peroxide to yield either oxygen or superoxide anion before returning ARP to its native form and completing the cycle.

According to Arnao et al., (1990b), a system without aromatic substrate would not reach the steady state at any time, leading to a total enzyme inactivation. This was confirmed by their observation, where repeated scans were performed, and a progressive decrease of compound III was observed, with a compensatory increase of compound P-670.

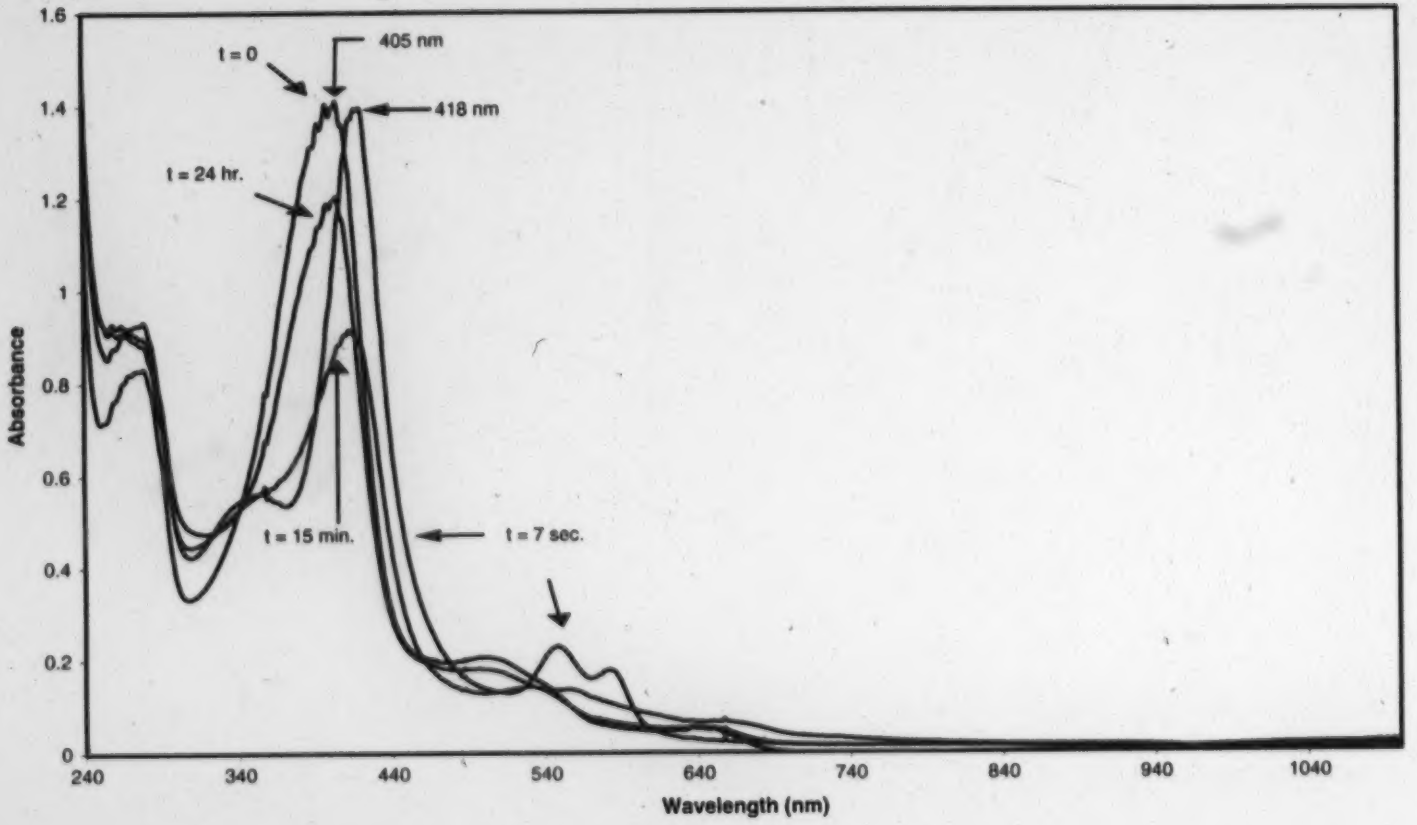

Figure 4-12. Spectral analysis of ARP, before and after the addition of 4 mM H_2O_2.

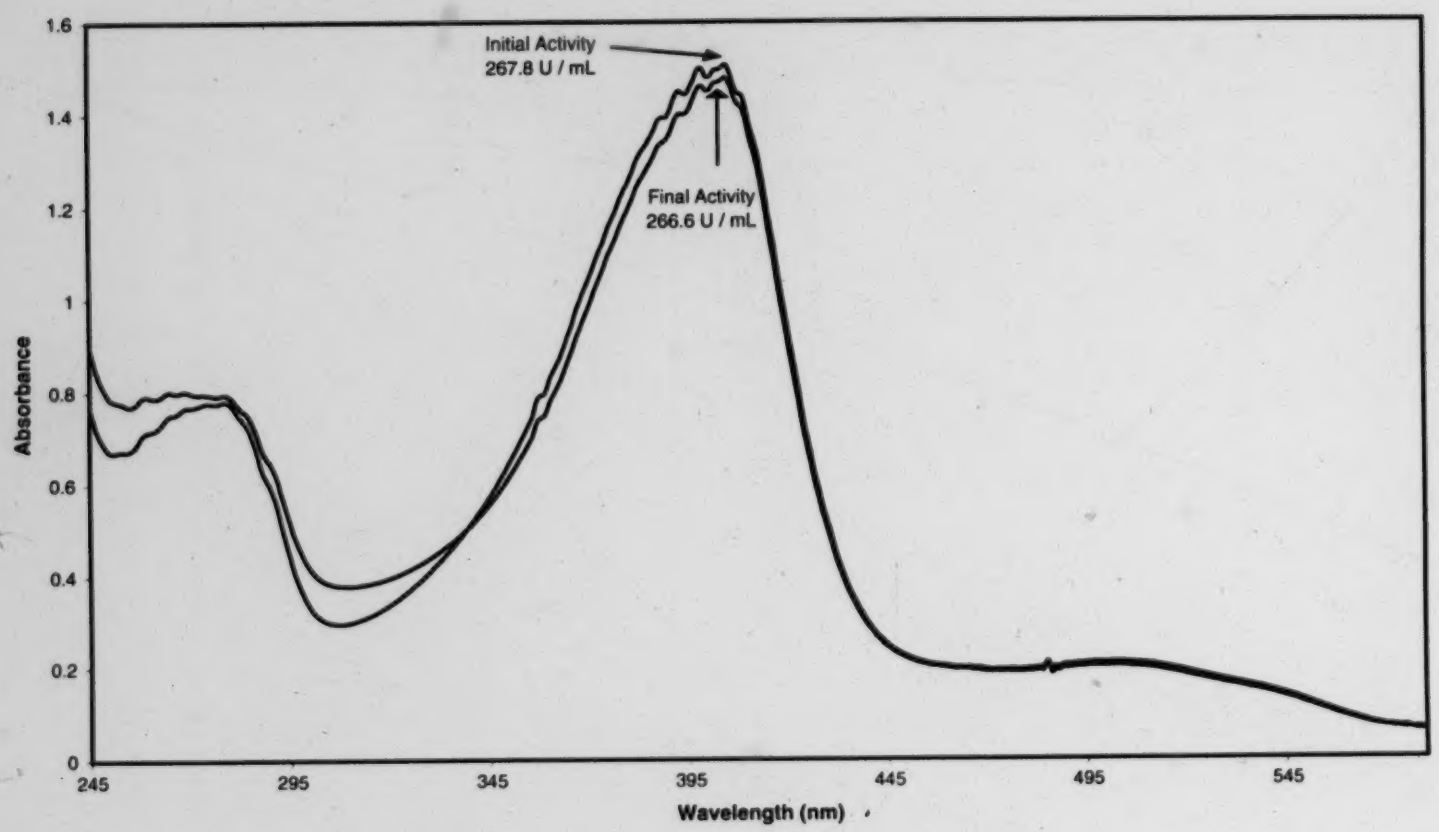

Figure 4-13. Detail of initial and final spectra for ARP with 0.25 mM initial H_2O_2.

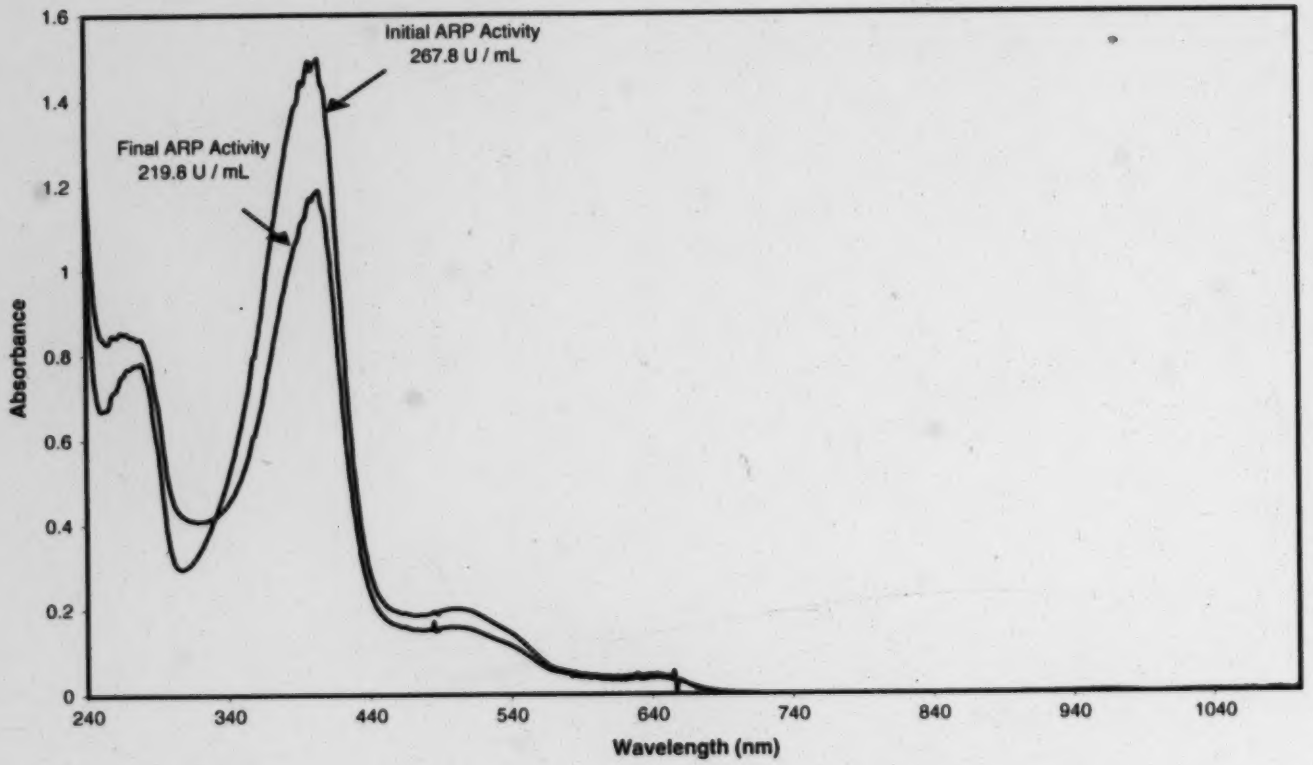

Figure 4-14. Initial and final spectra for ARP with 4 mM initial H_2O_2.

As can be observed in the previous Figures, no traces of the formation of compound P-670 were observed during this investigation with 0.25 and 4 mM peroxide. Other tests (data not shown) with higher peroxide concentrations (the highest when 20 µL of concentrated peroxide was added to the cuvette) were performed to find out if P-670 was formed, with no trace of the formation of this compound, corroborating Han's (1998) observations.

Even though the formation of compound P-670 has been apparently observed only with HRP, a general assumption on its formation has been adopted for all other peroxidases, but with the results obtained during this investigation, some of the proposed side reactions relating to inactivation could not apply to ARP (or some other peroxidases, such as CiP, CMP, or SBP). This would discard equation (2.11) from the reactions proposed by Arnao *et al.*, (1990a), and their statement about total enzyme inactivation (mentioned before).

Because of this divergence in one of the inactivation mechanisms, possible comparisons and results published between different peroxidases could be wrong. For example, Han (1998) found a disagreement between the simplified model developed by Buchanan and Nicell (1997) for HRP that predicted that the ARP enzyme would remain in the compound III form for over 60 minutes, and the result already mentioned with ARP. Han (1998) stated that a deficiency in the model or the method of parameter estimation was responsible for the divergence on results; Wright compared SBP inactivation over time with the results obtained by Nicell *et al.*, (1993b), Arnao *et al.*, (1990b), and Baynton *et al.*, (1994) using HRP, concluding that its inactivation was greater (with the same initial peroxide concentration) than that of SBP, and many other cases found in the literature. So care must be taken from now on, since the results from this research demonstrated (for ARP at least) that the same inactivation mechanisms can not be taken for granted for all peroxidases, and individual studies on each peroxidase must be performed to clarify this issue.

Nonetheless, as previously described, not all the activity was recovered on the samples. This indicates that as the initial peroxide is increased, part of the enzyme remains in an inactive form that seems to be permanent, or at least that regenerates very slowly to the native enzyme form and could not be observed during the timeframe here employed. A possible explanation is the formation of an intermediate containing some form of the enzyme and H_2O_2 (since the final activity seemed to depend on the initial peroxide). Arnao *et al.*, (1990b) reported that the formation of compound P-670 is preceded by the generation of several intermediate compounds, such as P-965 and P-940. These intermediates could not be responsible for the diminished activity, since no traces of them were found. Going back to the full cycle depicted in Figure 2-3, one possibility is that the complex formed by compound I and peroxide ($E_{I} \cdot H_2O_2$), for some reason, did not converted completely to native form. The second alternative (even though its existence has not been confirmed) is the presence of compound ($E_{II} \cdot H_2O_2$) [refer to equation 2.9]. Even though no traces of either compound I or II were found to corroborate this assumption, based on their known characteristic spectral absorbance (compound I with peak absorbance at 402 and 658 nm, and compound II at 419, 530, and 554 nm), the likelihood that these complexes could be formed, and hence, the reason for the observed unexplained inactivation of the enzyme is still possible, since no documentation exists describing their characteristic absorbance.

In Figure 4-15 and Figure 4-16 it can be appreciated that the final absorbance (in both cases) is higher within the absorbance range from 236 to 336 nm approximately. However, this higher absorbance observed does not seem to represent faithfully the lost in absorbance at the main peaks at 405 nm found between initial and final activity of ARP (as previously observed in Figure 4-13 and Figure 4-14). A slight difference in the measured activity units absorbed of these final spectrums from Figure 4-15 and Figure 4-16, and the similarity on the wavelength range suggest that other unknown compound(s) might be present in the reaction.

Further studies are required to identify if the measures obtained are reliable and repeatable, or if any interference was present which caused that occurrence, and if correct, identify which compound(s) absorb light at those wavelengths, to verify the speculations made here regarding the inactive form(s) produced.

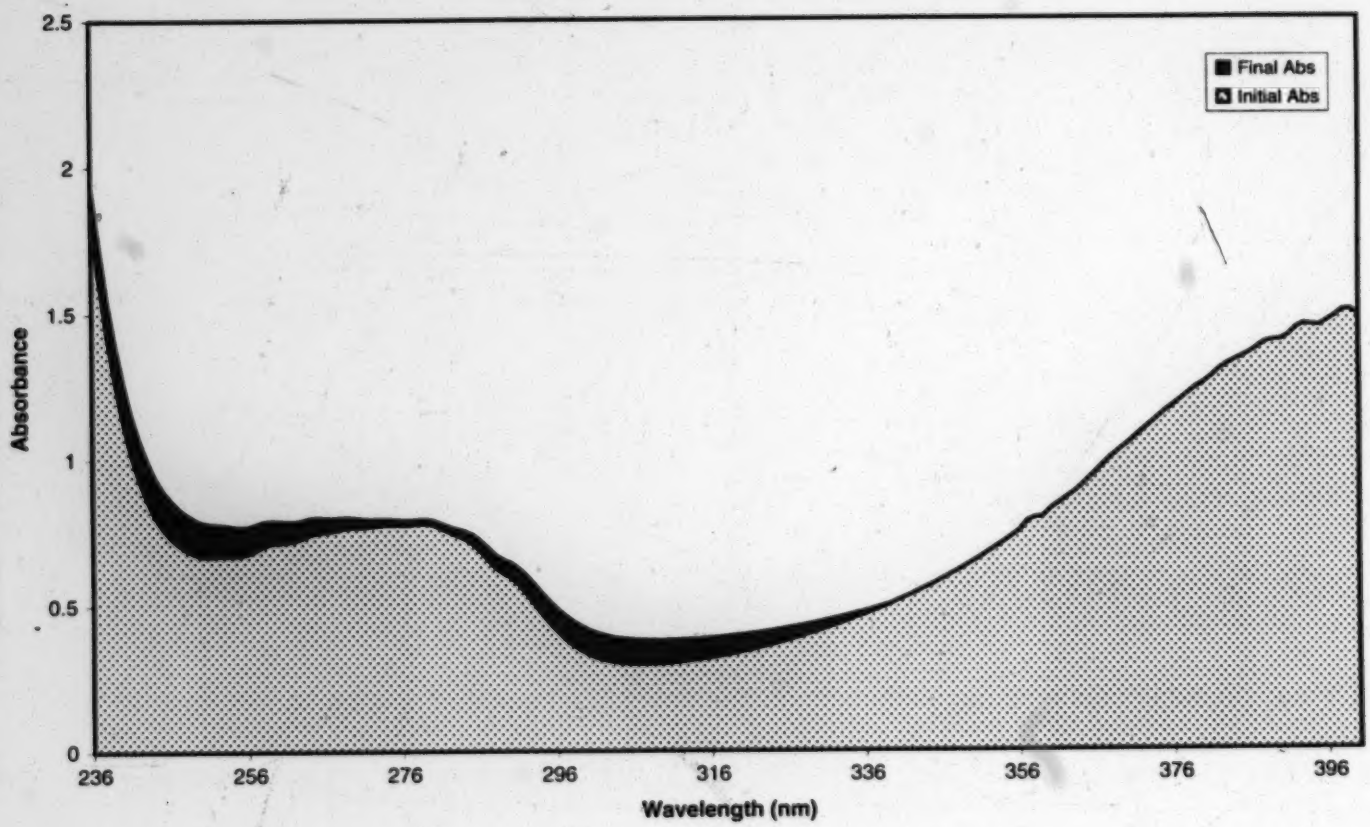

Figure 4-15. Final spectra observed for inactive ARP compound with 0.25 mM initial peroxide.

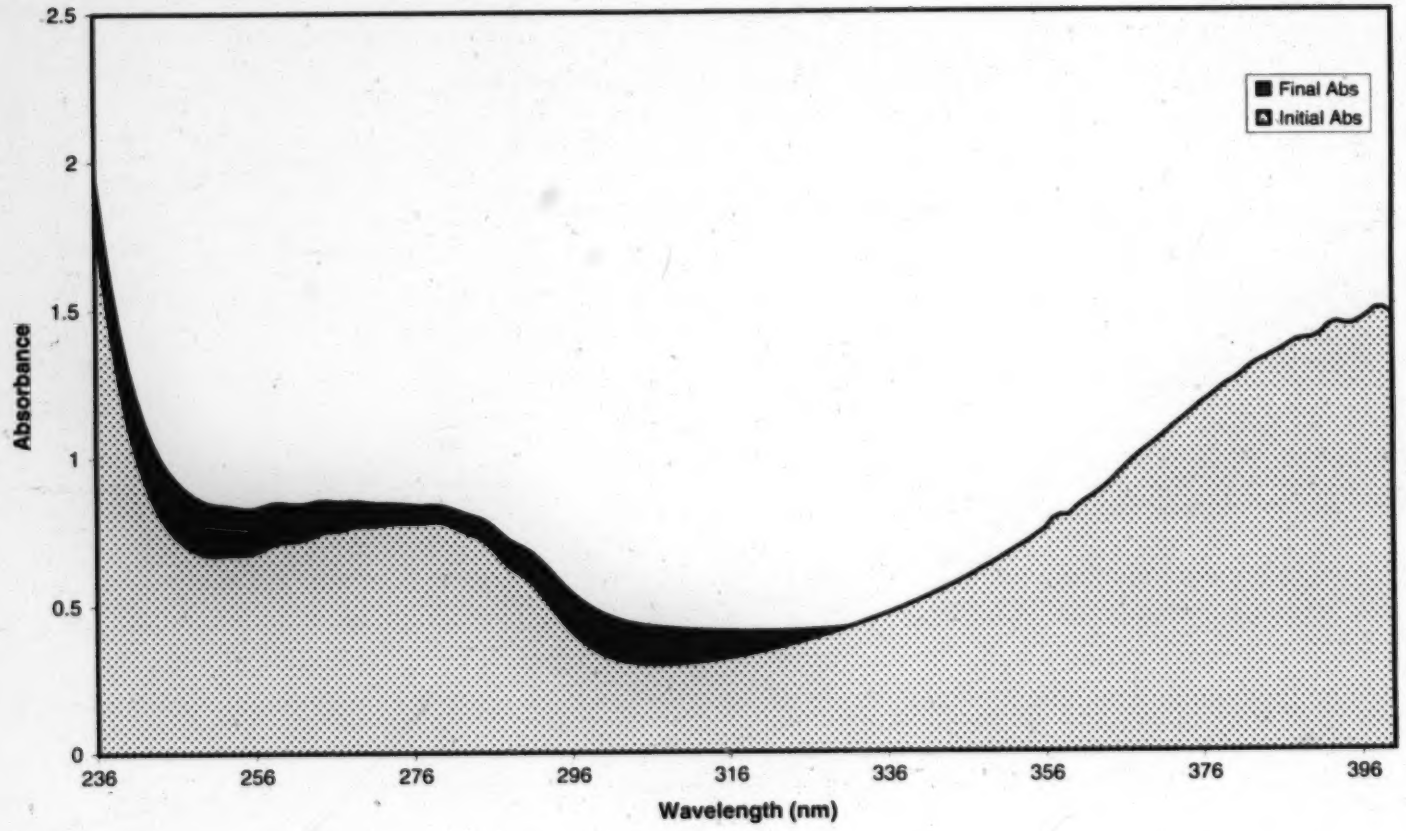

Figure 4-16. Detail of the final spectra observed for inactive ARP compound with 4 mM initial peroxide.

5. SUMMARY AND CONCLUSIONS

5.1. Stoichiometry

Attempts to model stoichiometry based on initial concentrations of enzyme, hydrogen peroxide, and/or phenol were unfruitful. The inconsistency encountered between the theoretical and measured stoichiometry seems to be dependent not only on these parameters, but some other mechanisms and complicated interactions that affect its value, such as the return of non-precipitated polymers to the active site, which can react again to form higher polymers, and their potential affinity with the enzyme to return to it, consequently increasing the observed stoichiometry. The randomness of the different dimers formed may also be affecting the consumption of peroxide per mole of phenol removed. However, as observed during this research and in many other cases, and with different peroxidases, a tendency towards a 1:1 stoichiometry was observed with all the batch tests, as peroxide dose was increased. A first assumption considered, is that from Nicell *et al.*, 1992, who explained that the increasing values in stoichiometry are caused by an increase in peroxide consumption due to larger polymer formation. However, the results obtained in this work point to another direction. The increased consumption of hydrogen peroxide was apparently related to formation of compound III and other inactive forms of the enzyme, involving peroxide, as will be mentioned in the following sections. Furthermore, the stoichiometry observed, apart from emphasizing the use of peroxide in the formation of inactive enzyme compounds, showed that a linear model is not suitable for describing the relationship between peroxide consumed and phenol precipitated from the solution, as it is with HRP.

5.2. Step Addition of Hydrogen Peroxide

Based on the turnovers observed for different enzyme concentrations from previous tests, the results suggest that phenol removal is linearly correlated to the initial concentration of enzyme present in the reaction mixture. Since the turnover values were very close in magnitude, an attempt to increase phenol removal based on step addition of

enzyme would be useless. Thus, step addition of peroxide was recommended, with some observed improvement in phenol removal. This improvement in phenol removal can be correlated with the enzyme inactivation mentioned in the previous section. When sufficient time was provided, the non-permanent inactive forms of the enzyme (i.e. compound III) were allowed to return to the native form, hence removing more phenol from the solution.

These experiments also assisted to emphasize that the main mechanism of inactivation was caused mainly by other reason than that of free radicals in the presence of PEG, since a higher instantaneous concentration of free radicals would supposedly inactivate more enzyme, which in turn, was not observed.

5.3. Spectrophotometric Measurements

Spectrophotometric measurements of native enzyme, and with the addition of peroxide were fundamental to consolidate the assumption of enzyme inactivation. With ARP and peroxide only, no free radicals were present in the reaction mixture, and the initial and final activity of the enzyme varied depending on the initial quantity of peroxide provided, indicating a lower final activity as peroxide concentration was raised. Since the only compound present was peroxide (other than the enzyme), and based on the side reactions proposed by Arnao *et al.* 1990a, compound III and peroxide-enzyme complexes were the only possibilities of enzyme intermediates that could be formed.

Spectral scans also indicated that the formation of P-670 did not occur with ARP in the presence of high concentrations of peroxide and in the absence of a reducing substrate, as observed with HRP. This suggests that the mechanism(s) of inactivation may differ for the various peroxidases studied to date.

Finally, unknown inactive forms of enzyme were observed to be formed during the reaction of ARP with peroxide, and further research is necessary to clarify this issue, and identify them.

6. RECOMMENDATIONS

6.1. Stoichiometry

Further research is needed to identify the most probable dimers formed during phenol oxidation by ARP and identify their affinity to return to the active site. This would help to comprehend the use of more hydrogen peroxide in the formation of higher polymers during the reaction.

6.2. Step Addition of Peroxide (Reactor Operation)

As observed during this research, step addition of hydrogen peroxide increased the removal of phenol, by allowing compound III to return to the normal enzyme cycle and removing more phenol. Thus, further investigation, changing the mode of peroxide addition (i.e. continuous addition over extended periods of time, longer reaction times between step addition) should be studied to find a better way to minimize the formation of the observed inactive forms caused by peroxide.

6.3. Spectrophotometric Measurements

Further work is needed to identify the unknown apparent permanently inactive complexes formed during the reaction of ARP and peroxide. Extended observation time is suggested to notice if those apparent inactive forms are in fact permanent, or they only require more time to return to the native enzyme form. Repetition of spectrophotometric measurements regarding the possible inactive compound(s) found within the absorbance range from 236 to 336 nm are suggested in order to clarify if these observations have any relevance concerning enzyme inactivation, or any kind of interference on the measures.

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A. APPENDIX A

ANALYTICAL METHODS

A.1. Chemicals

The following is a list of the chemicals used during this research and their molecular weights. The list is provided to facilitate the visualization of the calculations of reagent preparation in subsequent subsections of this Appendix.

Chemical	Formula	Molecular Weight (g/mol)
Certified ACS Sodium Bicarbonate	$NaHCO_3$	84.01
Certified ACS Sodium Phosphate Monobasic	$NaH_2PO_4 - H_2O$	137.99
Certified ACS Dibasic Anhydrous Sodium Phosphate	Na_2HPO_4	141.96
Certified ACS Potassium Ferricyanide	$K_3Fe(CN)_6$	329.26
Certified ACS Hydrogen Peroxide, 30%	H_2O_2	34.01
4-aminoantipyrine (4-AAP)	$C_{11}H_{13}N_3O$	203.25
Certified ACS Solid Phenol	C_6H_5O	94.11

A.2. *Arthromyces ramosus* Peroxidase Activity Assay

A colorimetric assay involving the reaction of excess phenol, 4-aminoantipyrine (4-AAP), hydrogen peroxide (H_2O_2), and rate limiting ARP in a monobasic-dibasic sodium phosphate buffer pH 7.4 was used to measure the amount of active enzyme present in a sample. In this assay, the free radicals combine with 4-AAP to form a red quinoneimine dye that absorbs light at a peak wavelength of 510 nm. The color generation during the first seconds is proportional to the rate of enzymatic H_2O_2 consumption, which in turn is proportional to the concentration of active enzyme in the sample (Wright, 1995).

The assay consists of 10 mM phenol, 2.4 mM AAP, 0.2 mM H_2O_2, in a 0.1 M monobasic-dibasic sodium phosphate (NaPP) buffer pH 7.4, and the enzyme sample, that must not exceed a concentration of 0.05 U/mL in order to maintain a linear relationship over the 1-minute duration of this assay.

A.2.1. Reagents

a) 0.2 M monobasic sodium phosphate

Dilute 27.6 g of monobasic sodium phosphate with ultra pure water to make 1 L.

b) 0.2 M dibasic sodium phosphate (anhydrous)

Dilute 28.4 g of dibasic sodium phosphate with ultra pure water to make 1 L.

c) 0.1 M NaPP Buffer (pH 7.4)

Add 19 mL of 0.2 M monobasic sodium phosphate to 81 mL of 0.2 M dibasic sodium phosphate and dilute to 200 mL with ultra pure water.

d) 20 mM Phenol

Dissolve 941.1 mg of phenol to 500 mL of 0.1 M NaPP buffer (pH 7.4).

e) 9.6 mM 4-aminoantipyrine (4-AAP)

Dissolve 390 mg of 4-AAP to 200 mL of 0.1 M NaPP buffer (pH 7.4). Store in refrigerator.

f) 2.0 mM H_2O_2

Dilute 113.4 µL of 30% (w/v) hydrogen peroxide to 100 mL with ultra pure water (10 mM). Mix well and withdraw 20 mL and dilute to 100 mL with ultra pure water (2.0 mM). This reagent has to be prepared daily.

A.2.2. Procedure

The volumes of buffer and enzyme sample should be combined to give a total assay volume of 1 mL. Thus, in a semi-micro cuvette place the following reagents:

- 500 µL of 20 mM phenol.
- 250 µL of 9.6 mM 4-AAP.
- 100 µL of 2.0 mM H_2O_2.
- 0 to 100 µL of 0.1 M NaPP (pH 7.4).
- 50 to 150 µL of enzyme sample.

Upon addition of the ARP, the cuvette has to be immediately covered and inverted at least 3 times prior to being placed in the spectrophotometer. The change in absorbance at 510 nm is monitored for at least one minute following reaction initiation.

A.2.3. Calculations

One unit of activity is defined as the number of micromoles of peroxidase converted per minute at 25 °C and pH 7.4. The enzyme activity is obtained from the average slope (absorbance units per minute) within the linear range. Thus, the activity in the cuvette in units of mol min^{-1} mL^{-1} (i.e. U/mL) is calculated from:

$$Activity\ in\ cuvette\ (U/mL) = \frac{slope\,(au/\min)}{6280\,(au \cdot L/mol)} \times \frac{10^6\,\mu mol}{mol} \times \frac{1\,L}{1000\,mL}$$

where *au* represents activity units, and 6280 $au \cdot L/mol$ relates the color development to peroxide consumption.

Furthermore, the sample activity has to be calculated according to:

$$Sample\ activity\ (U/mL) = Activity\ in\ cuvette(U/mL) \times \frac{1000\ \mu L}{vol.sample\ \mu L} \times Dilution^{3}$$

A.3. Total Phenol Assay

This assay measures the concentration of phenol in a sample using a colourimetric method. The phenol in the sample reacts with 2.08 mM 4-AAP in the presence of 8.34 mM potassium ferricyanide, which generates a yellowish color. After waiting for 8 to 10 minutes for total color development, the absorbance is measured at 510 nm. The total concentration of phenol in the cuvette is then determined using a calibration curve (as depicted in Figure 3.2). This assay is a modification of the direct photometric method, 5530 D, which is a standard analytical procedure for phenols (APHA, 1995). This modified assay permits measurement of higher phenol concentrations than the standard method, while using smaller sample volumes, by using higher concentrations of 4-AAP and potassium ferricyanide reagents.

A.3.1. Reagents

a) 0.25 M Sodium bicarbonate ($NaHCO_3$)

Dissolve 21.0 g of sodium bicarbonate with ultra pure water to make 1 L.

b) 83.4 mM Potassium ferricyanide

Dissolve 13.73 g of potassium ferricyanide using 0.25 M of $NaHCO_3$ to 500 mL and store in refrigerator.

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[3] Be aware that the sample activity has to be multiplied by the dilution factor (if any) used to maintain an enzyme concentration below or at the 0.05 U/mL required.

c) 20.8 mM 4-aminoatipyrine (4-AAP)

Dissolve 2.114 g of AAP using 0.25 M of $NaHCO_3$ to 500 mL Store in refrigerator.

d) 1.0 mM Phenol

Dissolve 47.055 mg of phenol to 500 mL using 0.1 M NaPP buffer (pH 7.4). With this stock solution make standard solutions ranging from 0 to 1 mM.

A.3.2. Calibration Curve Procedure

Mix the following reagents in the order stated:

- 700 µL of 0.25 M sodium bicarbonate.
- 100 µL of standard.
- 100 µL of 20.8 mM AAP solution.
- 100 µL of 83.4 mM potassium ferricyanide.

The assay sample volume must be 1 mL. The phenol concentration in the semi-micro cuvette must not exceed 0.1 mM. Invert several times the cuvette to mix the reagents and place in the spectrophotometer. Record the absorbance at 510 nm after full color is developed (within 8 to 10 minutes). Perform the measurements in triplicates for all standards. Plot the absorbance versus phenol concentration, calculating the slope of the line by linear regression.

A.3.3. Phenol Assay Procedure

In a semi-micro cuvette combine the following reagents to have a final volume of 1 mL:

- 0 to 700 µL of 0.25 M sodium bicarbonate.
- 100 to 800 µL of sample (not exceeding 0.1 mM of Phenol in cuvette)[4]
- 100 µL of 20.8 mM AAP solution.
- 100 µL of 83.4 mM potassium ferricyanide.

Wait for 8 to 10 minutes for full-color development and read the absorbance at 510 nm.

A.3.4. Calculations

The concentration of phenol in the cuvette is determined from the calibration curve, and the phenol concentration of the sample is obtained from:

$$[Phenol]_{sample} = [Phenol]_{cuvette} \times \frac{1000\ \mu L}{sample\ volume\ (\mu L)} \times Dilution$$

[4] Dilute the sample as required if the concentration of phenol will exceed 0.1 mM in the cuvette.

A.4. Hydrogen Peroxide Assay

Hydrogen peroxide concentration is measured using an end-point colourimetric assay that uses 10 mM phenol and 2.4 mM 4-AAP as ARP substrates at pH 7.4. Hydrogen peroxide must be the limiting substrate, thus the maximum concentration in the cuvette should not exceed 50 µM. When the maximum color is developed, the absorbance at 510 nm is converted to the concentration of hydrogen peroxide in the cuvette using a calibration curve.

A.4.1. Preparation of reagents

a) 0.1 M Phosphate buffer (pH 7.4)
Mix 19 mL of 0.2 M monobasic sodium phosphate with 81 mL of 0.2 M dibasic sodium phosphate and dilute to 200 mL with ultra pure water.

b) 20 mM phenol
Dissolve 941.1 mg of solid phenol to 500 mL of phosphate buffer (pH 7.4).

c) 9.6 mM 4-AAP
Dissolve 390 mg to 200 mL of phosphate buffer (pH 7.4). Store in refrigerator.

d) Stock ARP (1 mg/mL)
Dissolve 10 mg of solid ARP to 10 mL using phosphate buffer pH 7.4. Accuracy in the preparation of this stock solution is not required since the only requisite is excess enzyme to reach the endpoint.

e) 10 mM hydrogen peroxide

Dilute 113.4 µL of 30% w/v hydrogen peroxide to 100 mL with ultra pure water. From this 10 mM stock H_2O_2 make standard solutions with concentrations ranging from 0 to 0.5 mM.

A.4.2. Calibration Curve Procedure

In a semi-micro cuvette place the following reagents:

- 500 µL of 20 mM phenol.
- 250 µL of 9.6 mM 4-AAP.
- 100 µL of stock ARP.
- 50 µL of 0.1 M NaPP buffer (pH 7.4).
- 100 µL of standard peroxide solution.

Invert the cuvette several times and place in the spectrophotometer. Wait until the maximum amount of color has developed and record the absorbance at 510 nm. Repeat the same procedure for all peroxide standards prepared, using triplicate measurements.

Note that the assay sample volume must be 1 mL and that the peroxide concentration in the cuvette must not exceed 50 µM. Make the calibration curve by plotting absorbance versus peroxide concentrations in the cuvette.

A.4.3. Assay Procedure

Place the following reagents in a semi-micro cuvette for a final volume of 1 mL:

- 500 µL of 20 mM phenol.
- 250 µL of 9.6 mM 4-AAP.
- 100 µL of stock enzyme.
- 0 to 100 µL of 0.1 NaPP buffer (pH 7.4).
- 50 to 150 µL of sample.

Measure the maximum absorbance at 510 nm. Since the peroxide concentration should not exceed 50 μM, use more NaPP buffer and less sample volume if necessary (or dilute the sample as required) but always maintaining a total volume of 1 mL. The H_2O_2 concentration in the cuvette can be thus obtained from the calibration curve shown in Figure 3.3.

A.4.4. Calculations

The peroxide concentration in the sample is calculated from:

$$[H_2O_2]_{sample} = [H_2O_2]_{cuvette} \times \frac{1000\ \mu L}{Sample\ volume\ (\mu L)}$$

B. APPENDIX B

B.1. Batch Reactor Stoichiometry Data

Displayed data in Tables B-1 to B-12 contained 400 mg/L PEG and 7 U/mL (371.4 nM) ARP.

Table B-1. Batch reactor data with 1.04 mM initial phenol.

	Test 1		
Initial H_2O_2 (mM)	Abs. @ 510 nm	Phenol removed (mM)	Stoichiometry
0	1.041	0.000	0.000
0.025	1.006	0.036	0.695
0.05	0.985	0.057	0.878
0.1	0.907	0.135	0.741
0.25	0.700	0.342	0.732
0.5	0.400	0.641	0.780
1	0.006	1.035	0.966

Table B-2. Batch reactor data with 1.04 mM initial phenol.

	Test 2		
Initial H_2O_2 (mM)	Abs. @ 510 nm	Phenol removed (mM)	Stoichiometry
0	1.063	0.000	0.000
0.025	0.995	0.047	0.532
0.05	0.958	0.084	0.596
0.1	0.829	0.213	0.470
0.25	0.722	0.320	0.782
0.5	0.400	0.641	0.780
1	0.006	1.035	0.966

Table B-3. Batch reactor data with 1.04 mM initial phenol.

	Test 3		
Initial H_2O_2 (mM)	Abs. @ 510 nm	Phenol removed (mM)	Stoichiometry
0	1.022	0.000	0.000
0.025	0.983	0.059	0.424
n/a	n/a	n/a	n/a
0.1	0.890	0.152	0.659
0.25	0.697	0.345	0.725
0.5	0.400	0.641	0.780
1	0.003	1.038	0.963

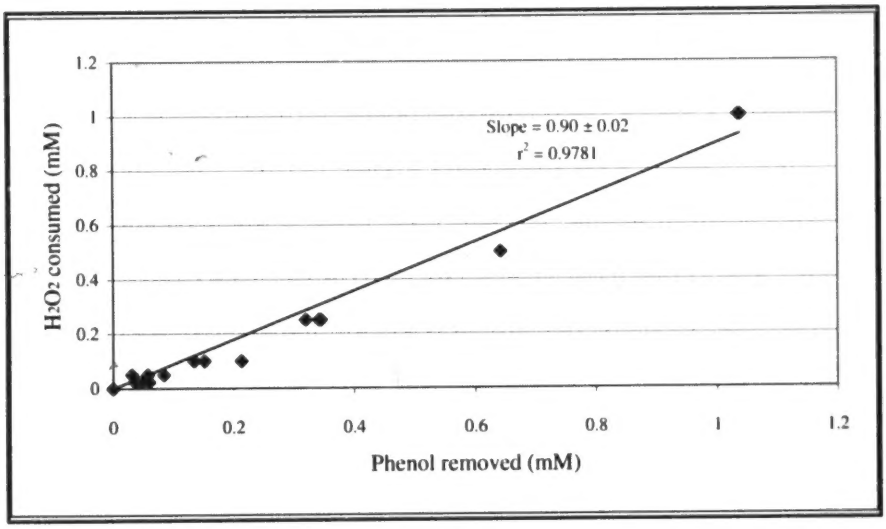

Figure B-1. Stoichiometry data from Tables B-1 to B-3 corresponding to 1.04 mM initial phenol.

Table B-4. Batch reactor data with 2.16 mM initial phenol.

	Test 1		
Initial H_2O_2 (mM)	Abs. @ 510 nm	Phenol removed (mM)	Stoichiometry
0.000	1.091	0.000	0.000
0.050	0.904	0.348	0.144
0.100	0.892	0.372	0.269
0.250	0.782	0.592	0.422
0.500	0.627	0.902	0.554
1.000	0.343	1.469	0.681
1.500	0.081	1.993	0.753
2.000	0.006	2.143	0.933

Table B-5. Batch reactor data with 2.16 mM initial phenol.

	Test 2		
Initial H_2O_2 (mM)	Abs. @ 510 nm	Phenol removed (mM)	Stoichiometry
0	1.058	0.000	0.000
0.05	0.900	0.356	0.140
0.1	0.906	0.344	0.290
0.25	0.813	0.530	0.472
0.5	0.635	0.886	0.564
1	0.352	1.451	0.689
1.5	0.084	1.987	0.755
2	0.004	2.147	0.932

Table B-6. Batch reactor data with 2.16 mM initial phenol.

	Test 3		
Initial H_2O_2 (mM)	Abs. @ 510 nm	Phenol removed (mM)	Stoichiometry
0	1.086	0.000	0.000
0.05	0.918	0.320	0.156
0.1	0.875	0.406	0.246
0.25	0.807	0.542	0.461
0.5	0.628	0.900	0.556
1	0.343	1.469	0.681
1.5	0.084	1.987	0.755
2	0.003	2.149	0.931

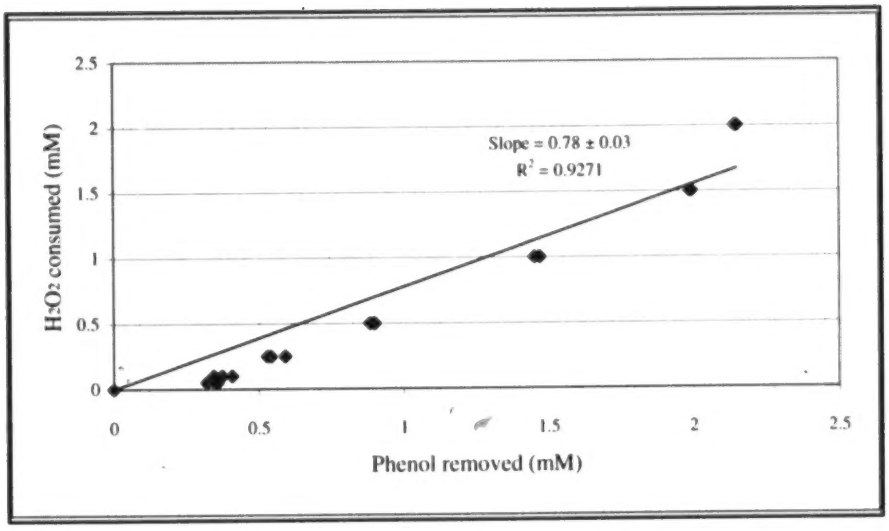

Figure B-2. Stoichiometry data from Tables B-4 to B-6 corresponding to 2.16 mM initial phenol.

Table B-7. Batch reactor data with 4.40 mM initial phenol.

	Test 1		
Initial H_2O_2 (mM)	Abs. @ 510 nm	Phenol removed (mM)	Stoichiometry
0	1.109	0.000	0.000
0.1	1.069	0.128	0.782
0.25	0.988	0.452	0.554
0.5	0.946	0.619	0.807
1	0.778	1.291	0.775
2	0.448	2.609	0.766
4	0.005	4.380	0.913

Table B-8. Batch reactor data with 4.40 mM initial phenol.

	Test 2		
Initial H_2O_2 (mM)	Abs. @ 510 nm	Phenol removed (mM)	Stoichiometry
0	1.105	0.000	0.000
0.1	1.062	0.156	0.642
0.25	1.022	0.316	0.792
0.5	0.967	0.535	0.934
1	0.775	1.303	0.768
2	0.449	2.605	0.768
4	0.003	4.388	0.912

Table B-9. Batch reactor data with 4.40 mM initial phenol.

	Test 3		
Initial H_2O_2 (mM)	Abs. @ 510 nm	Phenol removed (mM)	Stoichiometry
0	1.089	0.000	0.000
0.1	1.045	0.224	0.447
0.25	0.991	0.440	0.569
0.5	0.944	0.627	0.797
1	0.784	1.267	0.789
2	0.443	2.629	0.761
4	0.002	4.392	0.911

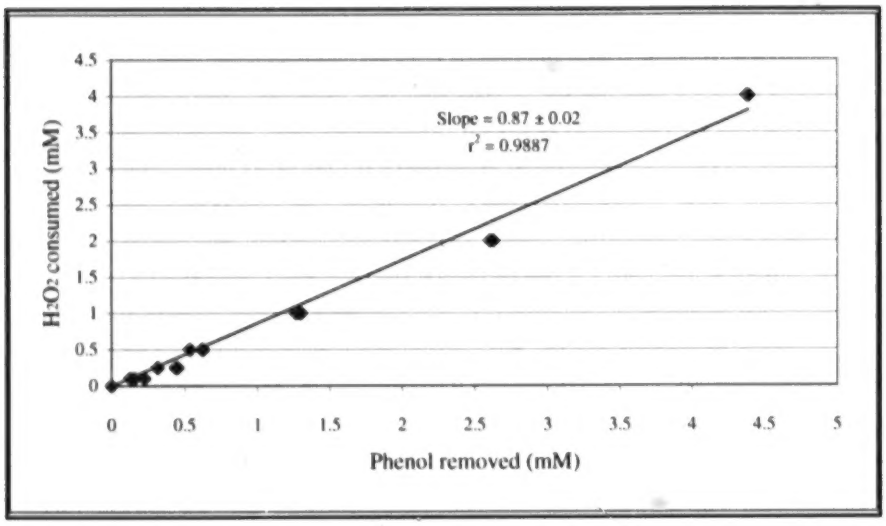

Figure B-3. Stoichiometry data from Tables B-7 to B-9 corresponding to 4.40 mM initial phenol.

Table B-10. Batch reactor data with 8.03 mM initial phenol.

	Test 1		
Initial H_2O_2 (mM)	Abs. @ 510 nm	Phenol removed (mM)	Stoichiometry
0	0.999	0.000	0.000
0.2	0.973	0.258	0.774
0.4	0.962	0.346	1.155
0.8	0.840	1.321	0.605
2	0.675	2.640	0.758
4	0.348	5.253	0.761
8	0.005	7.995	1.001

Table B-11. Batch reactor data with 8.03 mM initial phenol.

	Test 2		
Initial H_2O_2 (mM)	Abs. @ 510 nm	Phenol removed (mM)	Stoichiometry
0	1.011	0.000	0.000
0.2	0.977	0.226	0.883
0.4	0.961	0.354	1.129
0.8	0.843	1.297	0.617
2	0.665	2.720	0.735
4	0.385	4.958	0.807
8	0.005	7.995	1.001

Table B-12. Batch reactor data with 8.03 mM initial phenol.

	Test 3		
Initial H_2O_2 (mM)	Abs. @ 510 nm	Phenol removed (mM)	Stoichiometry
0	1.006	0.000	0.000
0.2	0.977	0.226	0.883
0.4	n/a	n/a	n/a
0.8	n/a	n/a	n/a
2	0.685	2.560	0.781
4	0.372	5.062	0.790
8	0.001	8.000	1.000

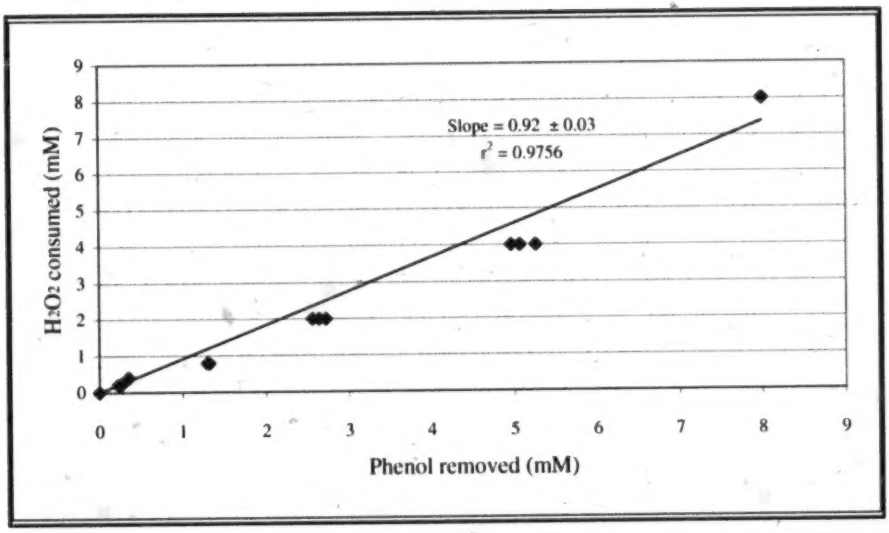

Figure B-4. Stoichiometry data from Tables B-10 to B-12 corresponding to 8.03 mM initial phenol.

B.2. Batch Reactor Data for Catalytic Turnovers

Table B-13. Data for turnovers capacity of ARP, at pH 7.0 and 400 mg/L PEG.

Test 1						
		Abs. @ 510 nm		(mM)		
ARP (U/mL)	ARP (M)	Initial Phenol	Initial H_2O_2	Phenol removed	H_2O_2 consumed	Turnovers
0	0	1.053	0.267	0.000	0.000	0
0.3	1.629E-08	0.788	0.221	1.069	0.621	65632
0.4	2.172E-08	0.649	0.198	1.625	0.944	74797
0.5	2.715E-08	0.553	0.198	2.008	0.944	73967
0.6	3.258E-08	0.411	0.153	2.576	1.574	79056
0.7	3.801E-08	0.304	0.120	3.003	2.037	79011
0.8	4.344E-08	0.229	0.075	3.303	2.667	76034
0.9	4.887E-08	0.129	0.057	3.703	2.919	75763
1	5.430E-08	0.091	0.045	3.854	3.087	70983
2	1.086E-07	0.009	0.019	4.182	3.452	38509
3	1.629E-07	0.003	0.005	4.206	3.648	25820
4	2.172E-07	0.000	0.001	4.218	3.704	19420
5	2.715E-07	0.001	0.000	4.214	3.718	15521
6	3.258E-07	0.004	0.000	4.202	3.718	12898
7	3.801E-07	0.040	0.000	4.058	3.718	10677

Table B-14. Data for turnovers capacity of ARP, at pH 7.0 and 400 mg/L PEG.

Test 2						
		Abs. @ 510 nm		(mM)		
ARP (U/mL)	ARP (M)	Initial Phenol	Initial H_2O_2	Phenol removed	H_2O_2 consumed	Turnovers
0	0	1.049	0.283	0.000	0.000	0
0.3	1.629E-08	0.777	0.221	1.113	0.621	68331
0.4	2.172E-08	0.648	0.198	1.629	0.944	74981
0.5	2.715E-08	0.549	0.166	2.024	1.392	74556
0.6	3.258E-08	0.421	0.149	2.536	1.630	77829
0.7	3.801E-08	0.299	0.115	3.023	2.107	79537
0.8	4.344E-08	0.231	0.075	3.295	2.667	75850
0.9	4.887E-08	0.128	0.057	3.707	2.919	75844
1	5.43E-08	0.088	0.045	3.866	3.087	71204
2	1.086E-07	0.006	0.017	4.194	3.480	38619
3	1.629E-07	0.030	0.004	4.098	3.662	25157
4	2.172E-07	0.006	0.004	4.194	3.662	19310
5	2.715E-07	0.004	0.000	4.202	3.718	15477
6	3.258E-07	0.060	0.000	3.978	3.718	12211
7	3.801E-07	0.005	0.000	4.198	3.718	11045

Table B-15. Data for turnovers capacity of ARP, at pH 7.0 and 400 mg/L PEG.

Test 3						
		Abs. @ 510 nm		(mM)		
ARP (U/mL)	ARP (M)	Initial Phenol	Initial H_2O_2	Phenol removed	H_2O_2 consumed	Turnovers
0	0	1.065	0.246	0.000	0.000	0
0.3	1.629E-08	0.784	0.221	1.085	0.621	66614
0.4	2.172E-08	0.646	n/a	1.637	n/a	75349
0.5	2.715E-08	0.549	0.186	2.024	1.112	74556
0.6	3.258E-08	0.426	0.151	2.516	1.602	77216
0.7	3.801E-08	0.300	0.112	3.019	2.149	79432
0.8	4.344E-08	0.228	n/a	3.307	n/a	76126
0.9	4.887E-08	0.125	0.065	3.719	2.807	76090
1	5.43E-08	0.093	0.045	3.846	3.087	70836
2	1.086E-07	0.008	0.019	4.186	3.452	38545
3	1.629E-07	0.007	0.000	4.190	3.718	25721
4	2.172E-07	0.003	0.005	4.206	3.648	19365
5	2.715E-07	0.003	0.000	4.206	3.718	15492
6	3.258E-07	0.003	0.000	4.206	3.718	12910
7	3.801E-07	0.000	0.000	4.218	3.718	11097

B.3. Batch Reactor Data for Step Addition of Hydrogen Peroxide

Table B-16. Step addition of peroxide, day 1.

H_2O_2	4 mM		2/2 mM		1/1/1/1 mM	
	Abs @ 510 nm	Phenol remaining (mM)	Abs @ 510 nm	Phenol remaining (mM)	Abs @ 510 nm	Phenol remaining (mM)
Day 1	0.047	0.188	0.275	1.099	0.560	2.238
	0.066	0.264	0.302	1.207	0.560	2.238
	0.055	0.220	0.277	1.107	0.566	2.262
Avg	0.056	0.224	0.285	1.138	0.562	2.246

Table B-17. Step addition of peroxide, day 2.

H_2O_2	4 mM		2/2 mM		1/1/1/1 mM	
	Abs @ 510 nm	Phenol remaining (mM)	Abs @ 510 nm	Phenol remaining (mM)	Abs @ 510 nm	Phenol remaining (mM)
Day 2	0.075	0.300	0.072	0.288	0.275	1.099
	0.084	0.336	0.082	0.328	0.271	1.083
	0.062	0.248	0.078	0.312	0.285	1.139
Avg	0.074	0.294	0.077	0.309	0.277	1.107

Table B-18. Step addition of peroxide, day 3.

H_2O_2	4 mM		2/2 mM		1/1/1/1 mM	
	Abs @ 510 nm	Phenol remaining (mM)	Abs @ 510 nm	Phenol remaining (mM)	Abs @ 510 nm	Phenol remaining (mM)
Day 3	0.063	0.252	0.077	0.308	0.023	0.092
	0.089	0.356	0.084	0.336	0.019	0.076
	0.080	0.320	n/a	n/a	0.016	0.064
Avg	0.077	0.309	0.081	0.322	0.019	0.077

Table B-19. Step addition of peroxide, day 4.

H_2O_2	4 mM		2/2 mM		1/1/1/1 mM	
	Abs @ 510 nm	Phenol remaining (mM)	Abs @ 510 nm	Phenol remaining (mM)	Abs @ 510 nm	Phenol remaining (mM)
Day 4	0.089	0.356	0.090	0.360	0.040	0.160
	0.066	0.264	0.078	0.312	0.031	0.124
	0.083	0.332	0.078	0.312	0.018	0.072
Avg	0.079	0.317	0.082	0.328	0.030	0.119

Table B-20. Step addition of peroxide, time limiting, 1 hour.

H_2O_2	4 mM		2/2 mM		1/1/1/1 mM	
	Abs @ 510 nm	Phenol remaining (mM)	Abs @ 510 nm	Phenol remaining (mM)	Abs @ 510 nm	Phenol remaining (mM)
1 hour	0.083	0.332	0.406	1.622	0.728	2.909
	0.100	0.400	0.409	1.634	0.705	2.817
	0.088	0.352	0.416	1.662	0.704	2.813
Avg	0.090	0.361	0.410	1.640	0.712	2.846

Table B-21. Step addition of peroxide, time limiting, 2 hours.

H_2O_2	4 mM		2/2 mM		1/1/1/1 mM	
	Abs @ 510 nm	Phenol remaining (mM)	Abs @ 510 nm	Phenol remaining (mM)	Abs @ 510 nm	Phenol remaining (mM)
2 hours	0.076	0.304	0.071	0.284	0.402	1.606
	0.094	0.376	0.063	0.252	0.401	1.602
	0.086	0.344	0.065	0.260	0.402	1.606
Avg	0.085	0.341	0.066	0.265	0.402	1.605

Table B-22. Step addition of peroxide, time limiting, 3 hours.

H_2O_2	4 mM		2/2 mM		1/1/1/1 mM	
	Abs @ 510 nm	Phenol remaining (mM)	Abs @ 510 nm	Phenol remaining (mM)	Abs @ 510 nm	Phenol remaining (mM)
3 hours	0.084	0.336	0.067	0.268	0.135	0.539
	0.085	0.340	0.072	0.288	0.127	0.507
	0.079	0.316	n/a	n/a	0.136	0.543
Avg	0.083	0.330	0.070	0.278	0.133	0.530

Table B-23. Step addition of peroxide, time limiting, 4 hours.

H_2O_2	4 mM		2/2 mM		1/1/1/1 mM	
	Abs @ 510 nm	Phenol remaining (mM)	Abs @ 510 nm	Phenol remaining (mM)	Abs @ 510 nm	Phenol remaining (mM)
4 hours	0.081	0.324	0.068	0.272	0.046	0.184
	0.092	0.368	0.069	0.276	0.059	0.236
	0.086	0.344	0.060	0.240	0.060	0.240
Avg	0.086	0.345	0.066	0.262	0.055	0.220

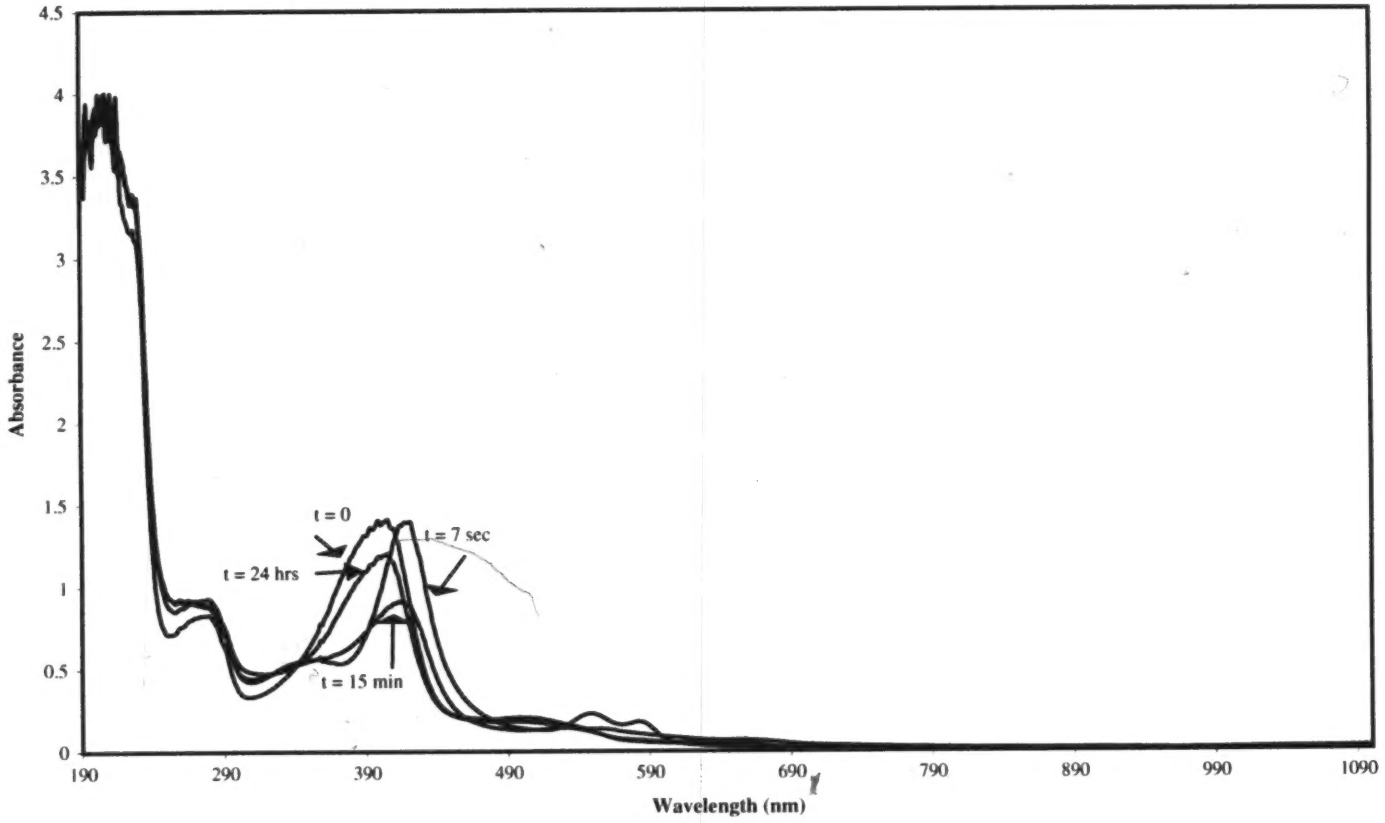

Figure B-5. Complete spectral scan for Figure 4-12.

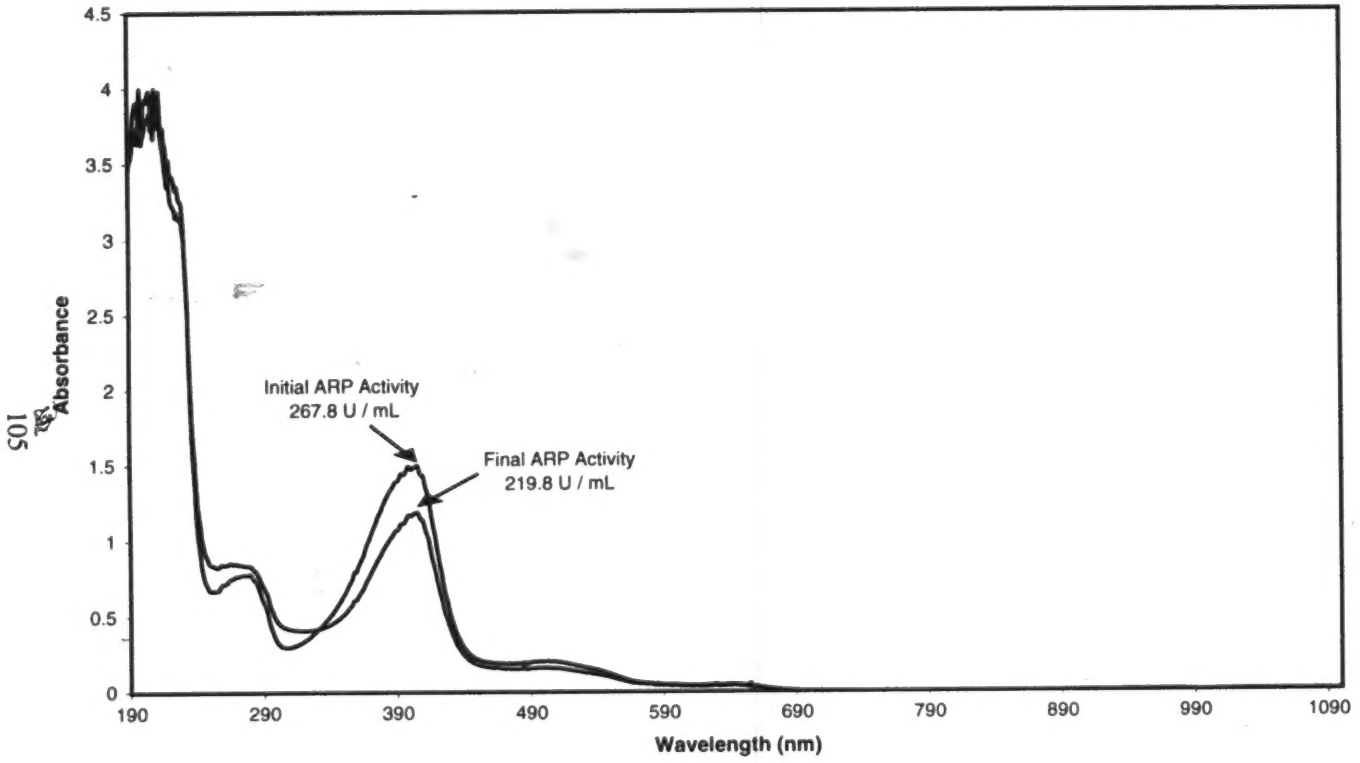

Figure B-6. Complete spectral scan for Figure 4-14.